AD_____

Award Number: W81XWH-06-1-0454

TITLE: The Role of ABC Proteins in Drug Resistant Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Jacqueline K. Lekostaj

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

REPORT DATE: April 2008

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-04-2008	Annual Summary	15 MAR 2006 - 14 MAR 2008

4. TITLE AND SUBTITLE

The Role of ABC Proteins in Drug Resistant Breast Cancer Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0454

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jacqueline K. Lekostaj

E-Mail: jkl25@georgetown.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Georgetown University
Washington, DC 20057

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This predoctoral training grant, supported by the Department of Defense Breast Cancer Research Program (BCRP) of the Office of the Congressionally Directed Medical Research Programs (CDMRP), aims to study the possible role of ABC transporters in pleitropic drug resistance, using a combination of molecular biological, biochemical, and biophysical methods. The second year of funding was spent working with a member of a drug/metabolite transporter superfamily called the Plasmodium falciparum Chloroquine Transporter (PfCRT). While PfCRT is known to be the main molecular determinant of chloroquine resistance, there is only indirect proof of its interaction with the drug. Therefore, I have employed a methodology commonly applied to drug resistance proteins (such as human P-glycoprotein) and labeled PfCRT with a photoaffinity drug analogue. A manuscript is currently in preparation detailing my results.

15. SUBJECT TERMS
Drug resistance, drug transporters, heterologous expression, photoaffinity labeling

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	34	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

INTRODUCTION

Members of the ATP-binding cassette (ABC) superfamily are proteins composed of transmembrane domains (TMDs) and nucleotide binding domains (NBDs), usually organized in single or tandem cassettes of 6 TM helices followed by a single NBD. Most ABC proteins are membrane transporters which act to translocate their substrates between various compartments. A number of drug resistance-conferring ABC proteins, including human P-glycoprotein (Pgp), have been proposed to function as drug transporters, using the energy from ATP hydrolysis to catalyze drug efflux from a cell or cellular compartment containing the relevant drug target, thereby promoting drug resistance. Although a large number of the known ABC proteins are active pumps (that is, they can transport against the electrochemical gradient of the substrate), there are several examples exhibiting divergent functions. The human ABC50 is a dimeric ABC without TMDs that has nuclear targeting signals and might be involved in aminoacyl-tRNA binding. Also, the cystic fibrosis transmembrane conductance regulator (or CFTR) is a chloride channel, and the sulphonylurea receptors (SUR1 and SUR2) are intracellular ATP sensors that regulate the permeability of potassium channels (Klein et al 1999). This predoctoral training grant, supported by the Department of Defense Breast Cancer Research Program (BCRP) of the Office of the Congressionally Directed Medical Research Programs (CDMRP), aims to study the possible role of ABC transporters in pleitropic drug resistance, using a combination of molecular biological, biochemical, and biophysical methods.

BODY

In my first year of work on this project, I heterologously expressed and characterized the P-glycoprotein homologue, the *Plasmodium falciparum* Multidrug Resistance protein (PfMDR1). I found very little remarkable drug stimulation or inhibition of ATPase activity. The contribution of PfMDR1 to malarial drug resistance seems minimal, at best playing a modulatory role in the resistance pre-determined by other factors. Therefore, in the past year, I have focused on another protein more directly responsible for drug response, the *Plasmodium falciparum* Chloroquine Resistance Transporter (PfCRT) (Fidock *et al* 2000). While not a member of the ABC family, PfCRT is a membrane protein belonging to a drug/metabolite transporter superfamily. Previous work in our lab utilized heterologous expression of yeast-optimized PfCRT expressing a polyHistidine tag and a Biotin Acceptor Domain (BAD). Using equilibrium centrifugation assays, it was found that PfCRT binds tritiated chloroquine (CQ). Some small differences were observed in affinity between HB3 (chloroquine sensitive, CQS) and Dd2 (chloroquine resistant, CQR) PfCRT isoforms, which may have been caused by the mutations present in PfCRT (Zhang *et al* 2004). However, equilibrium binding experiments are tedious, expensive, and do not easily allow for examination of multiple isoforms or competition studies (e.g. determination of relative affinity for CQ vs. quinine (QN) vs. mefloquine (MQ), etc.).

Other labs have had great success characterizing the drug binding sites of Pgp using various radioactive and photoreactive compounds (Safa *et al* 1986, Bruggemann *et al* 1989, Bruggemann *et al* 1992, Morris *et al* 1994). We therefore designed and synthesized a CQ photoaffinity analogue (Figure 1). This probe (AzBCQ) places a per fluoro phenyl azido (pfpa) group at the terminal aliphatic nitrogen of CQ, via a 4 carbon ester linker. The molecule also includes a convenient biotin tag (can be quantified by avidin-HRP detection and can also be used to purify protein-drug conjugates). Both the azide and biotin groups are attached by flexible linkers whose lengths can be easily modified. Importantly, the essential pharmacophore of the drug is preserved, and the pKa of the side chain remains unaltered. Upon UV irradiation, the

aryl azide forms a reactive nitrene which can undergo carbon-hydrogen insertion with the protein. Similar strategies have been used to successfully characterize such diverse proteins as the human GLUT4 transporter (Yang *et al* 2002), the *E.coli* ATPase SecA (Musial-Siwek *et al* 2007), and proteins involved in malaria-infected RBC adherence (Gowda *et al* 2007).

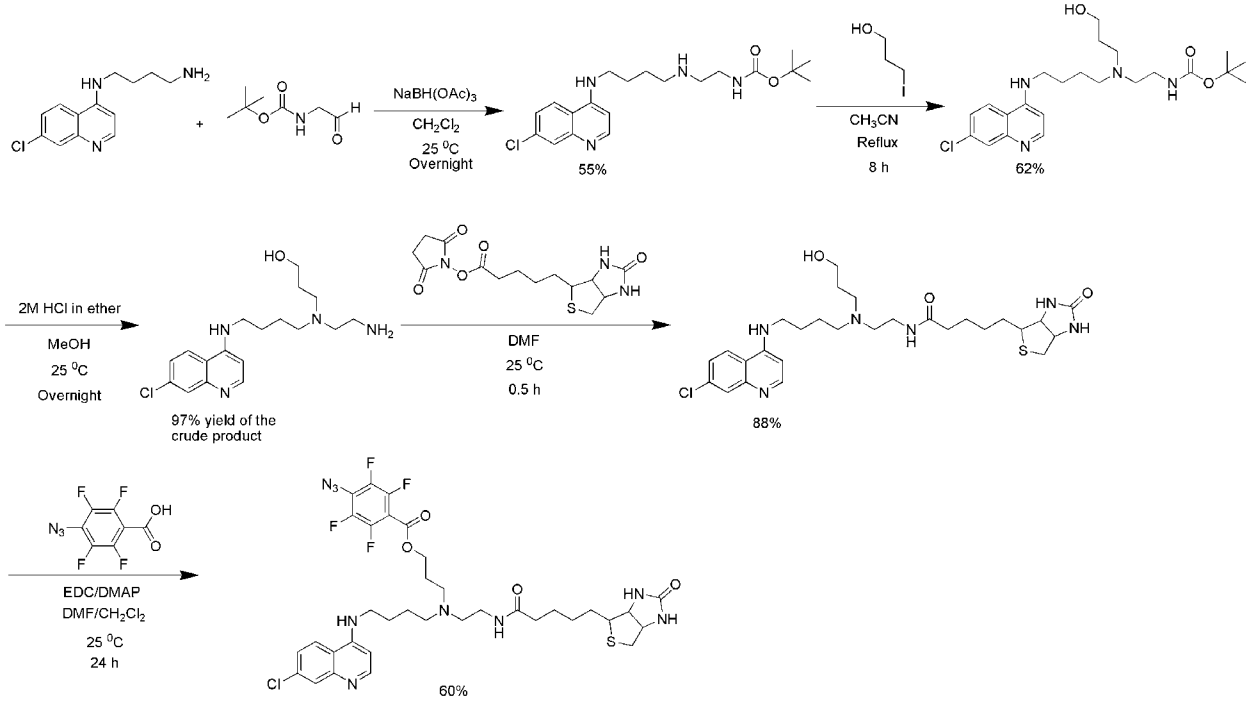


Figure 1. *Synthetic scheme for Azido-Biotinylated Chloroquine (AzBCQ)*

In order to test our yeast-expressed PfCRT for binding to this probe, I had to first remove the BAD from our constructs, leaving only the hexaHistidine tag. After inducing protein expression, I harvested the yeast membranes, partially purified the protein by affinity chromatography, and reconstituted the protein in proteoliposomes (PLs, Figure 2).

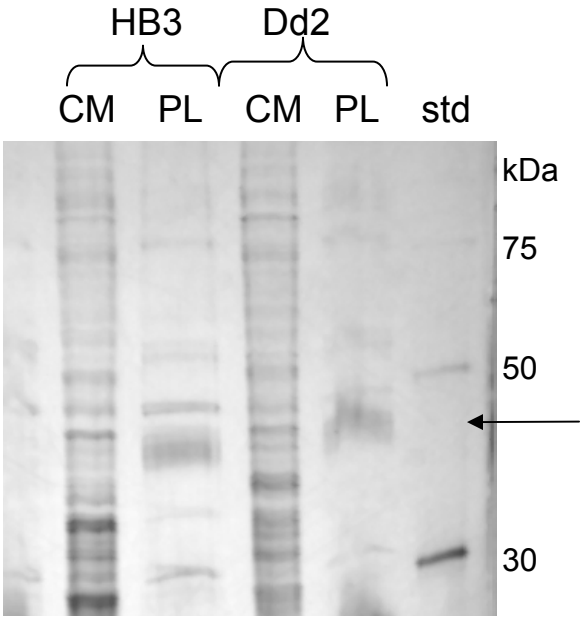


Figure 2 *Silver stain of crude membranes and proteoliposomes*. PfCRT was partially purified from crude membranes (CMs) and reconstituted into proteoliposomes (PLs). While not purified to homogeneity, contaminating yeast proteins are drastically reduced and PfCRT is significantly enriched. 1 ug protein was loaded per lane. Molecular weight standards (std) are in the rightmost lane and the position of PfCRT is indicated by the arrow.

The PLs were then tested for the ability to bind AzBCQ. Each photolabeling reaction was performed using 0.1 nmol PfCRT protein, and the other reagents were added in relative molar stoichiometries. The samples were then run on two parallel gels, transferred to PVDF, and then blotted for either biotin (which detects AzBCQ bound to protein) or the polyHis tag (which detects total amount of protein present). The bands were then analyzed by densitometry and the density of the biotin band was divided by the density of the polyHistidine band in order to normalize between lanes. This technique is demonstrated in Figure 4.

First, we tested a couple of basic experimental parameters in order to optimize binding conditions. A titration of probe concentration (Figure 3A) seems to show that AzBCQ has a slightly higher affinity for the HB3 isoform compared to Dd2. Half of the maximum labeling occurs at 11.1-fold molar excess of AzBCQ for HB3 compared to 19.8-fold for Dd2. The amount of labeling achieved plateaus by 5 minutes of UV irradiation (Figure 3B). Since PfCRT resides in the membrane of the digestive vacuole, and therefore is exposed to both the acidic compartment and the relatively neutral cytosol, we next tested the effect of pH on binding of the probe. As shown in Figure 4, AzBCQ binds both HB3 and Dd2 PfCRT isoforms better at lower pH.

Calculating the affinity of AzBCQ for PfCRT is difficult because the irreversible nature of the binding in the assay makes equilibrium conditions impossible. I have attempted to estimate an apparent affinity through competition studies with unlabeled quinoline drugs.

AzBCQ does appear to be functionally comparable to CQ, as labeling is readily diminished by increasing concentrations of CQ (Figure 5A). In terms of maximum binding, the competition is similar for both HB3 and Dd2 isoforms, with 50% knockdown occurring at 26.6- and 25.1- fold molar excess of unlabeled CQ. In live parasites, the CQR conferred by the Dd2 *Pfcrt* allele is reversed by verapamil (VPL) through an unknown mechanism. One possibility is that VPL associates with PfCRT and blocks it from transporting CQ out of the DV. Photolabeling with AzBCQ is competed by VPL (Figure 5B), but at much higher concentrations compared to CQ.

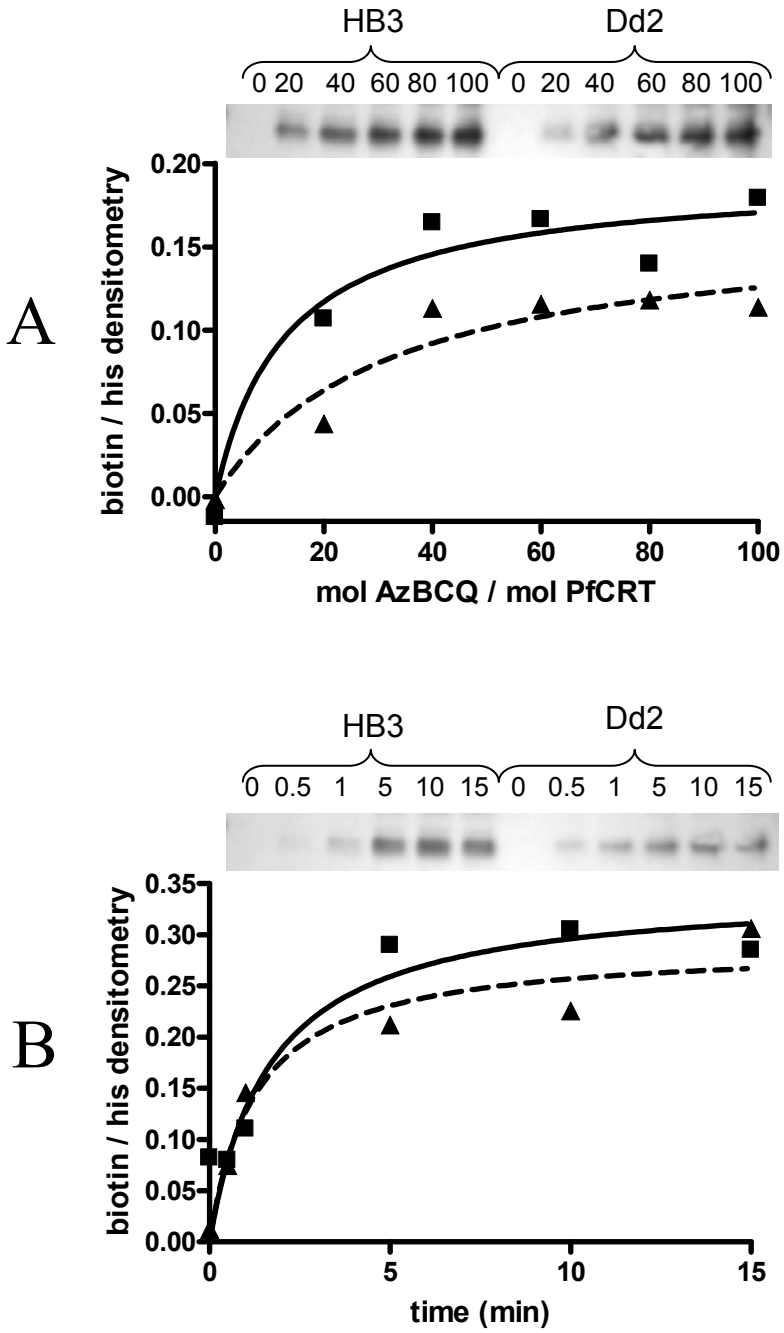


Figure 3. *AzBCQ labeling of HB3 and Dd2 PfCRT as a function of probe concentration and UV illumination time***.** (A) Normalized densitometry and avidin-HRP blot (inset) of HB3 (squares, solid line) and Dd2 (triangles, dashed line) PfCRT photolabeled with varying concentrations of AzBCQ at pH 5.4 and 10 min UV illumination time. (B) Normalized densitometry and avidin-HRP blot (inset) of HB3 (squares, solid line) and Dd2 (triangles, dashed line) PfCRT photolabeled with 50x molar AzBCQ at pH 5.4 and varying UV illumination time.

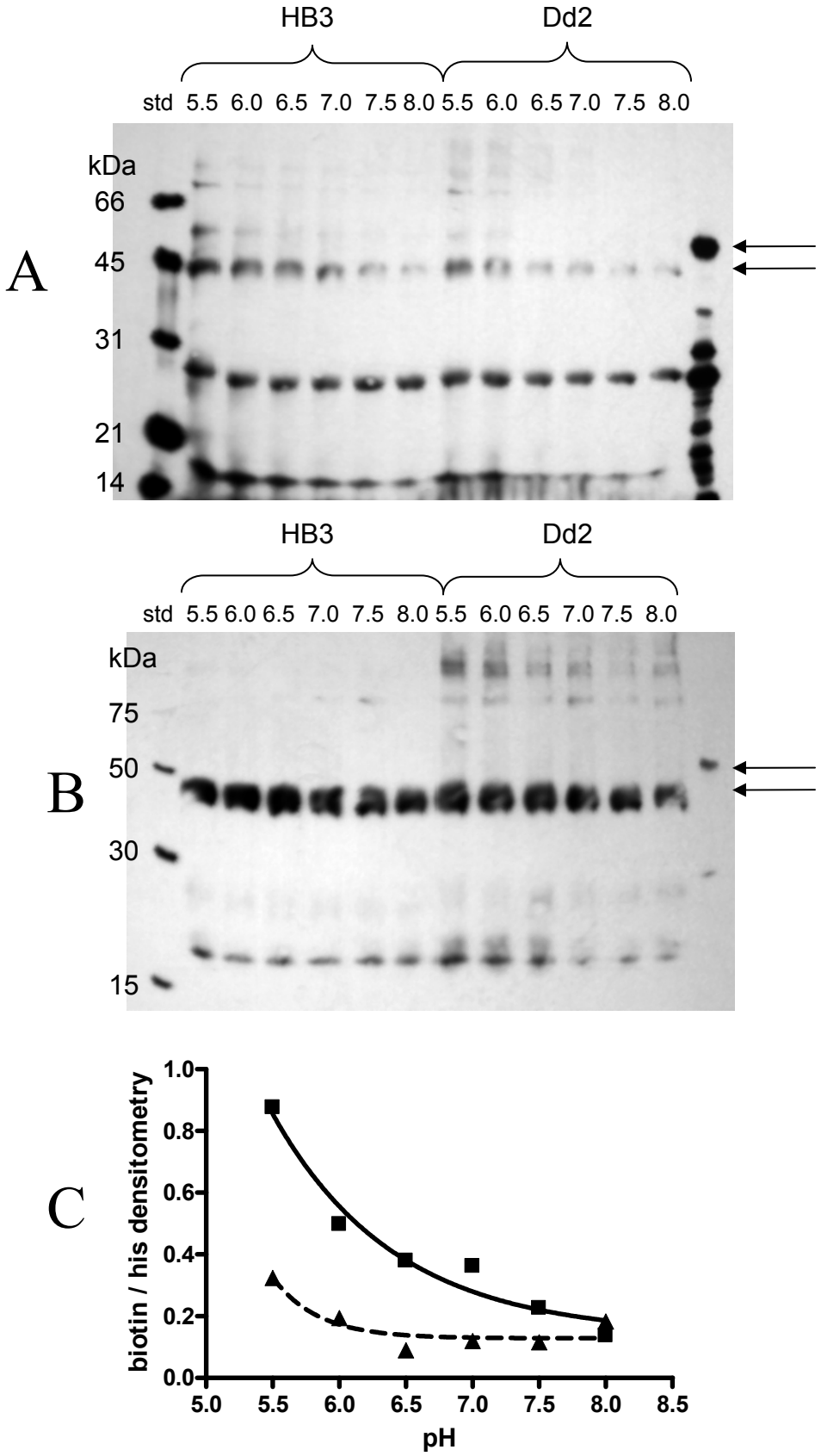
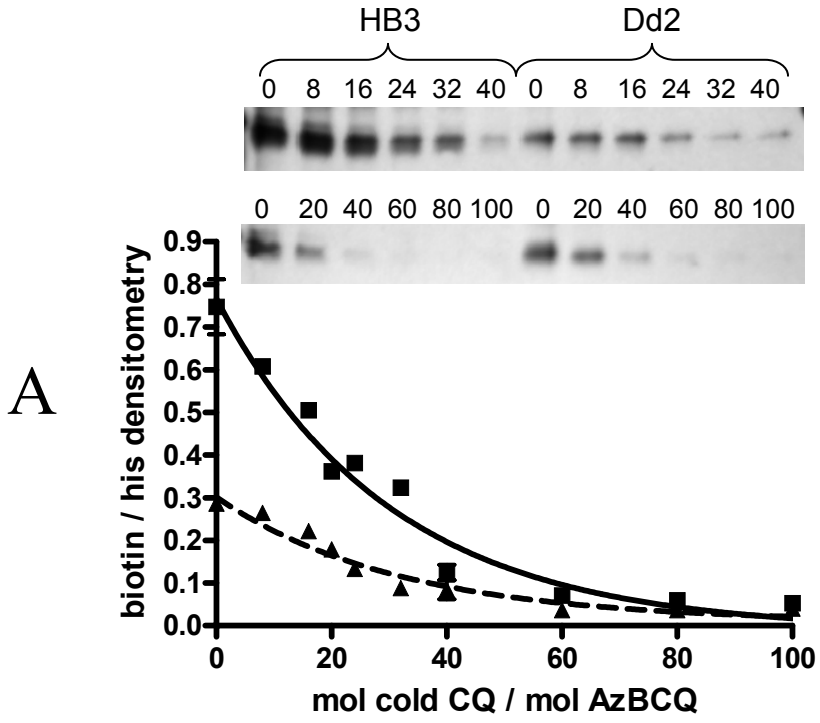


Figure 4. *AzBCQ labeling of HB3 and Dd2 PfCRT as a function of pH AzBCQ labeling***.** Avidin-HRP (A) blot of HB3 and Dd2 PfCRT photolabeled with increasing amounts of AzBCQ

at pH 5.4 and 10 min UV illumination. Avidin-HRP (A) and polyHis (B) blots of HB3 and Dd2 PfCRT were photolabeled with 50x molar AzBCQ at varying pH and 10 min UV illumination. The full, uncropped blots are shown, revealing specific binding of the probe despite contaminating proteins in the PLs. PfCRT tagged with both polyHis residues and a biotin acceptor domain (BAD, see reference 5) was run as a positive control (rightmost lane). Crt-His-BAD runs at ~57 kDa (upper arrow) whereas CRT-His runs at ~48 kDa (lower arrow). The band running at ~30 kDa is a naturally biotinylated yeast protein that is present even in the absence of AzBCQ. The normalized densitometry for HB3 (squares, solid line) and Dd2 (triangles, dashed line) is shown in (C).



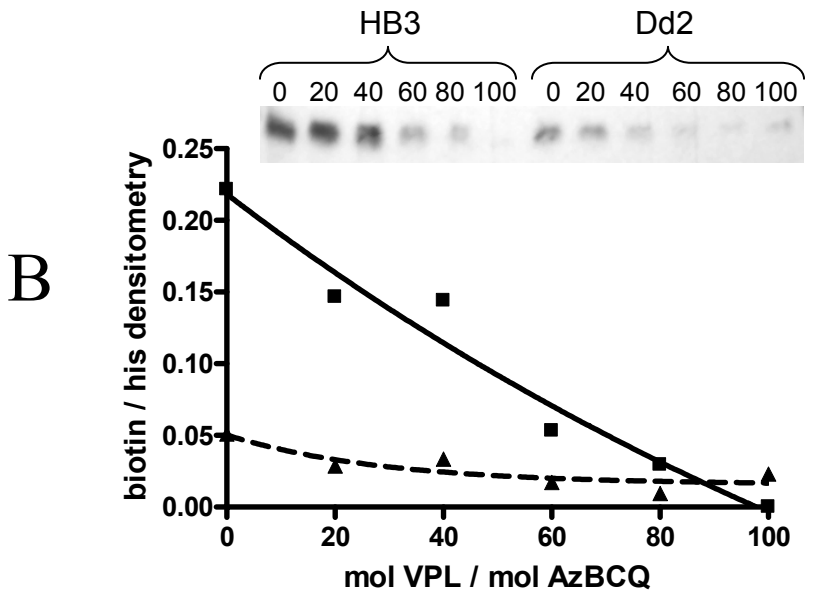


Figure 5. *Competition of AzBCQ labeling of HB3 and Dd2 PfCRT with "cold" drugs.* (A) Normalized densitometry and avidin-HRP blots (inset) of HB3 (squares, solid line) and Dd2 (triangles, dashed line) PfCRT photolabeled with 50x AzBCQ at pH 5.2 and 10 min UV illumination in the presence of varying amounts of unlabeled CQ. Competition is almost linear within the lower range of CQ concentrations (dotted lines). The equation of the line from 0-40 is $y = -0.015x + 0.73$ with $r^2=0.959$ for HB3 (squares, solid line) and $y = -0.0058x + 0.30$ with $r^2=0.946$ for Dd2 (triangles, dashed line). (B) Normalized densitometry and avidin-HRP blot (inset) of HB3 (squares, solid line) and Dd2 (triangles, dashed line) PfCRT photolabeled with 50x AzBCQ at pH 5.2 and 10 min UV illumination in the presence of varying amounts of unlabeled VPL. Half of the maximum binding is achieved at 42.4- and 37.7-fold excess VPL for HB3 and Dd2, respectively.

KEY ACCOMPLISHMENTS

Research Accomplishments

- Creation of yeast strains expressing PfCRT with a PolyHis tag
- Development and optimization of a photolabeling assay procedure
- Proof that chloroquine directly interacts with the protein thought to be responsible for resistance to the drug

Training Accomplishments

- Attendance of numerous seminars concerning breast cancer presented by the Lombardi Comprehensive Cancer Center and the Georgetown University Department of Oncology
- Participation in weekly seminars and journal clubs addressing both cancer-specific and basic science research, intended to stimulate awareness of other areas of research and new laboratory techniques that may inspire my own research

REPORTABLE OUTCOMES

- Published Article: Amoah LE, Lekostaj JK and Roepe PD. (2007) Heterologous expression and ATPase activity of mutant vs. wildtype PfMDR1 protein. *Biochemistry* 46(20):6060-73
- Published Article: Lekostaj JK, Amoah LE, and Roepe PD. (2008) "A single S1034C mutation confers altered drug sensitivity to PfMDR1 ATPase activity that is characteristic of the 7G8 isoform." *Molecular & Biochemical Parasitology* 157: 107-111.
- Presentation: "A Novel Perfluorophenyl Azido-Chloroquine Probe that Efficiently Photolabels PfCRT" Woods Hole Molecular Parasitology Meeting, September 16-20, 2007
- Abstract/poster: "Photoaffinity Labeling of *P. falciparum* Chloroquine Resistance Transporter (PfCRT)" American Society of Tropical Medicine and Hygiene Annual Conference, November 4-8, 2007
- Presentation: "The Relative Contribution of ABC Transporters in Pleiotropic Drug Resistance" Georgetown University Tumor Biology Graduate Student Data Meeting, February 28, 2008

CONCLUSION

Over the past year, I have been working PfCRT, a member of a drug/metabolite transporter superfamily. I have utilized a photoaffinity analogue synthesized in-house by the Roepe lab to test for substrate binding to the protein. Binding of our probe is specific, which speaks to both the quality of our probe as well as the affinity of chloroquine for the protein. Binding is also pH dependent. With decreasing pH, we are titrating the amount of protonated chloroquine, so perhaps CRT only binds the charged species. pH is also titrating the charge on the amino acids of the protein, in particular that at the drug-resistance critical position 76 (at which HB3 is the charged lysine and Dd2 is the neutral threonine). Either of these interpretations may indicate that the probe is binding to the side of the protein that is disposed towards the DV in the parasite. Labeling of the probe can be competed by "cold" CQ, which

indicates that the two molecules share the same binding site. This is another validation of the quality of our probe. The competition of labeling by verapamil suggests that CRT may also interact with this compound, and that CQ and VPL share the same or at least partially overlapping recognition sites.

Currently, I am continuing work with AzBCQ labeling of PfCRT and PfMDR1. I already have expressed and purified several more isoforms, on which I have begun binding experiments. I have also been testing other antimalarials (QN, MQ, Artemisinin) for their ability to compete with AzBCQ. I am also testing AzBCQ binding to purified PfMDR1. However, my biggest project right now is mapping the AzBCQ binding site by mass spectrometry. I am hoping to be able to proteolyze the labeled protein and identify the segment or segments to which the probe is bound. In addition, my lab is planning to synthesize AzB-quinine and AzB-mefloquine and test these compounds for labeling of PfCRT and PfMDR.

REFERENCES

Bruggemann EP, Germann UA, Gottesman MM, Pastan I. (1989) Two different regions of P-glycoprotein [corrected] are photoaffinity-labeled by azidopine *Journal of Biological Chemistry* 264(26):15483-8

Bruggemann EP, Currier SJ, Gottesman MM, Pastan I. (1992) Characterization of the azidopine and vinblastine binding site of P-glycoprotein *Journal of Biological Chemistry* 267(29):21020-6

Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu AB, Naudé B, Deitsch KW, Su XZ, Wootton JC, Roepe PD, and Wellems TE. (2000) Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Molecular Cell* 6(4): 861–71.

Gowda AS, Madhunapantula SV, Achur RN, Valiyaveettil M, Bhavanandan VP, and Gowda DC. (2007). Structural basis for the adherence of Plasmodium falciparum-infected erythrocytes to chondroitin 4-sulfate and design of novel photoactivable reagents for the identification of parasite adhesive proteins. *Journal of Biological Chemistry* 282: 916-28.

Klein I, Sarkadi B, & Varadi A (1999) An inventory of human ABC proteins. *Biochimica et Biophysica Acta* 1461: 237-62.

Morris DI, Greenberger LM, Bruggemann EP, Cardarelli C, Gottesman MM, Pastan I, Seamon KB. (1994) Localization of the forskolin labeling sites to both halves of P-glycoprotein: similarity of the sites labeled by forskolin and prazosin. *Molecular Pharmacology* 46(2):329-37

Musial-Siwek M, Rusch SL, Kendall DA. (2007). Selective photoaffinity labeling identifies the signal peptide binding domain on SecA. *Journal of Molecular Biology* 365: 637-48.

Safa AR, Glover CJ, Meyers MB, Biedler JL, Felsted RL. (1986) Vinblastine photoaffinity labeling of a high molecular weight surface membrane glycoprotein specific for multidrug-resistant cells. *Journal of Biological Chemistry* 261(14):6137-40

Yang J, Hodel A, and Holman GD. (2002). Insulin and isoproterenol have opposing roles in the maintenance of cytosol pH and optimal fusion of GLUT4 vesicles with the plasma membrane. *Journal of Biological Chemistry* 277: 6559-66.

Zhang H, Paguio M, and Roepe P (2004) The antimalarial drug resistance protein Plasmodium falciparum chloroquine resistance transporter binds chloroquine. *Biochemistry* 43(26): 8290-6.

APPENDICES

Amoah LE, Lekostaj JK and Roepe PD. (2007) Heterologous expression and ATPase activity of mutant vs. wildtype PfMDR1 protein. *Biochemistry* 46(20):6060-73

Lekostaj JK, Amoah LE, and Roepe PD. (2008) "A single S1034C mutation confers altered drug sensitivity to PfMDR1 ATPase activity that is characteristic of the 7G8 isoform." *Molecular & Biochemical Parasitology* 157: 107-111.

Abstract: "Photoaffinity Labeling of *P. falciparum* Chloroquine Resistance Transporter (PfCRT)" American Society of Tropical Medicine and Hygiene Annual Conference, November 4-8, 2007

Heterologous Expression and ATPase Activity of Mutant versus Wild Type PfMDR1 Protein†

Linda E. Amoah,‡ Jacqueline K. Lekostaj,‡ and Paul D. Roepe*

Department of Chemistry, Department of Biochemistry, Cellular & Molecular Biology, Tumor Biology Program Lombardi Cancer Center, and Center for Infectious Disease, Georgetown University, 37th and O Streets, Washington, D.C. 20057

Received January 30, 2007; Revised Manuscript Received March 21, 2007

ABSTRACT: Mutation of the *P. falciparum* chloroquine resistance transporter (PfCRT) causes resistance to chloroquine (CQ) and other antimalarial drugs. Mutation and/or overexpression of one of the multidrug resistance protein homologues found in this malarial parasite (PfMDR1) may further modify or tailor the degree of multidrug resistance. However, considerable controversy surrounds the precise contribution of PfMDR1, in part because no direct biochemical studies of PfMDR1 have yet been possible. Using codon optimization and other principles, we have designed and constructed a yeast optimized version of the wild type *pfmdr1* gene and have successfully overexpressed PfMDR1 protein in *P. pastoris* yeast. The protein is well expressed in either full length form or as two separate half transporters, is well localized to the yeast plasma membrane and is fully functional as evidenced by ATPase activity measurements. We have also expressed mutants that have previously been hypothesized to influence drug resistance in parasites. Using purified plasma membrane fractions, we have analyzed antimalarial drug effects on ATPase activity for wild type versus mutant proteins. Relative to other ABCB transporters involved in drug resistance, PfMDR1 is unusual. It has similar pH, [ATP], and Mg^{++} dependencies for ATP hydrolysis, yet relatively high K_m and V_{max} values for ATP hydrolysis, and ATPase activity is only mildly stimulated by antimalarial drugs. The largest measured drug effect is for CQ (to which PfMDR1 is not believed to confer resistance), and it is strongly inhibitory for WT PfMDR1. Drug resistance associated PfMDR1 mutants show either elevated (Dd2 allele encoded) or reduced (7G8 allele) basal ATPase activity and different patterns of drug stimulation or inhibition, relative to WT PfMDR1. The Dd2 PfMDR1 isoform also shows a slightly more alkaline pH optimum. Surprisingly, verapamil alone (1−300 μM) does not significantly affect WT ATPase activity but inhibits the Dd2 isoform at 1 μM. These data should assist ongoing analysis of the contribution of PfMDR1 to antimalarial drug resistance.

Chloroquine resistance (CQR[1]) in *P. falciparum* malaria requires mutations in the parasite PfCRT protein (*1*−*3*). A number of mutant PfCRT alleles that confer CQR are known to exist, and *P. falciparum* strains harboring these continue to both spread and evolve around the globe. These strains have different geographic origin (e.g., Africa vs South America), and their precise sensitivity to specific antimalarial drugs varies, but in general, mutation of PfCRT confers an approximately 10-fold increase in CQ IC_{50} that can be (but is not always) reversed by sublethal doses of verapamil (VPL). CQR is typically accompanied by resistance to other drugs, particularly related quinoline-based compounds, such as quinine (QN), quinidine (QD), amodiaquine (AQ), and mefloquine (MQ). That is, CQR-conferring mutations in PfCRT are likely involved in conferring some degree of these pleiotropic "quinoline resistance" phenomena (*2*). However, there is mounting evidence that mutations in and/or alternate expression of other proteins further modulate or augment CQR conferred by PfCRT mutations, particularly high level MQR and/or QNR concomitant with CQR. These additional phenomena may also contribute to multidrug resistance (MDR[2]), meaning simultaneous resistance to multiple chemical classes of drugs, such as quinolines, acridines, reactive

† This work was supported by NIAID/NIH Grant RO1 AI056312.

* Corresponding author. Tel: 202 687 7300. Fax: 202 687 6209. E-mail roepep@georgetown.edu.

‡ These authors contributed equally to this work.

[1] Abbreviations: PfCRT, *Plasmodium falciparum* chloroquine resistance transporter; CQ, chloroquine; PfMDR1, *Plasmodium falciparum* multidrug resistance protein; WT, wild type; ABC, ATP-binding cassette; CQR, chloroquine resistance (resistant); VPL, verapamil; QN, quinine; QD, quinidine; AQ, amodiaquine; MQ, mefloquine; QR, quinoline resistance (pleiotropic quinoline resistance); MDR, multidrug resistance; Pgh1, P-glycoprotein homologue [PfMDR1]; PfNHE1, *Plasmodium falciparum* Na^+/H^+ exchanger 1; HF, halofantrine; ART, artemisinin; CQS, chloroquine sensitive; QNR, quinine resistance; PM, plasma membrane; NBD, nucleotide binding domain; TMD, transmembrane domain; ABCB, B subfamily ABC transporter; DV, digestive vacuole; Pgp, P-glycoprotein.

[2] Although used in a general sense, regardless of the system being examined (tumor cells, parasites, and bacteria), multidrug resistance refers to a spectrum of phenomena that may vary widely with regard to the drugs in question, the degree of resistance, and the number and type of genetic events linked to the resistance phenotype. The term is usually meant to indicate pleiotropic resistance to multiple chemical classes of drugs or multiple pharmacophores; thus, we use the term quinoline resistance [QR] when referring to genotypic or phenotypic features known to mediate resistance to multiple quinolines (CQ, QN, AQ, etc.) but for which relevance to MDR phenomena is less well understood.

10.1021/bi7002026 CCC: $37.00
Published on Web 05/01/2007

endoperoxides, and so forth (but neglecting antifolates, resistance to which is typically conferred by mutations in one or more pyrimidine pathway enzymes). These MDR linked phenomena include alternate expression and/or mutation of the *P. falciparum* human P-glycoprotein homologue PfMDR1 (also called Pgh1) and the *P. falciparum* sodium proton exchanger (PfNHE1 (*4*−*10*)).

A number of conflicting observations surround the putative link between PfMDR1 protein and antimalarial drug resistance. In the early 1990s, when altered expression and/or mutation of PfMDR1 was widely assumed to be the principal cause of *P. falciparum* CQR in much of the drug resistance literature, Wellems and colleagues showed that the chromosomal locus harboring PfMDR1 did not segregate with the CQR phenotype in progeny of a genetic cross (*6*, *7*). This argued for little-to-no role for PfMDR1 in CQR. Indeed, subsequently, mutations in another vacuolar membrane protein (PfCRT) were shown to be the primary cause of CQR in *P. falciparum* (*1*). However, the Cowman group published results arguing for a small, but measureable, effect on sensitivity to CQ, QN, MQ, HF, and ART due to three mutations in the C-terminal half of PfMDR1 (S1034C, N1042D, and D1246Y) (*8*). These mutations represent three of the four found in the *pfmdr1* allele for CQR strain 7G8 (South America). Reversion of the three (back to wild type S, N, and D) mildly decreased the degree of CQR for strain 7G8, whereas their introduction into a CQS strain conferred 2-fold resistance to QN and MQ, and mild increased susceptibility to HF and ART. Following this report, Sidhu et al. (*9*) performed similar experiments but noted only mild QNR upon introduction of the triple mutant and only in one of two transfected strains. Recent field data (*5*) suggest that overexpression but not necessarily the mutation of *pfmdr1* is more relevant to drug resistance observed in the field. This is consistent with some earlier results from Wirth, Cowman, and other laboratories with cultured parasite cell lines (*10*) as well as more recent analysis of drug induced effects on *pfmdr1* transcription (*11*). Perhaps multiple contributions of PfMDR1 to drug resistance are possible in a strain specific manner that depends upon the *pfcrt* allele that is present. Another hypothesis is that some mutant PfMDR1 proteins perform a function similar to that promoted by increased expression of the wild type. In sum, the role of PfMDR1 in antimalarial drug resistance is important but is likely relatively minor and critically depends on the presence of other mutations (e.g., PfCRT).

Further molecular level analysis of PfMDR1 function and of its precise contribution to antimalarial drug resistance is hampered by the protein's vacuolar membrane localization within an intracellular parasite. Clearly, further studies would benefit from the availability of more convenient experimental systems. However, there are significant challenges to high level heterologous expression of this protein that have not previously been met because of the very high AT content of the *pfmdr1* gene.

A previous study by Gros and co workers reported expression of the native *pfmdr1* cDNA in yeast on the basis of indirect evidence (*12*), but this paper was subsequently retracted (*13*). In hindsight, the very high AT content (75%) along with other features of the *pfmdr1* gene likely precluded efficient, stable expression (*14*). Indeed, no western blot confirmation of PfMDR1 expression was reported, and the published retraction (*13*) suggests that inadvertent false positive transfection with the PfMDR1 homologue STE6 was likely responsible for some, if not all, of the phenotypic features analyzed in the selected clones. Low levels of expression for some endogenous *P. falciparum* cDNAs (encoding smaller soluble proteins or soluble domains of larger proteins) have been reported, but to our knowledge, the combination of unusual gene structure, large size, and other features has prevented routine high level heterologous overexpression of large malarial parasite polytopic integral membrane proteins (*14*).

Therefore, similar to earlier work with PfCRT (*14*), we have back translated the PfMDR1 protein sequence and have designed a yeast optimized synthetic *pfmdr1* gene. We have fused this to poly His and biotin acceptor-encoding domains, and report stable high level, inducible overexpression of WT PfMDR1 protein in *P. pastoris* yeast. We are able to express the two half transporters as well as the full length version of the protein in biotinylated form and report similarly efficient expression of 3D7, Dd2, and 7G8 isoforms of PfMDR1. Because drug stimulation of ATPase activity is considered to be a hallmark of ABCB protein-mediated drug resistance phenomena, we compare the ATPase activities of these isoforms under various conditions to test hypotheses for their role in antimalarial drug resistance phenomena.

MATERIALS AND METHODS

Materials. *Pfu*Ultra was from Stratagene (La Jolla, CA). Streptavidin HRP and ECL detection reagents were from Amersham Biosciences (Piscataway, NJ). Prestained SDS−PAGE molecular markers were from Bio-Rad (Hercules, CA). The PentaHis HRP detection kit, mini-elute spin columns, and miniprep spin columns were from Qiagen (Valencia, CA). *Pichia* plasmids and expression reagents were from Invitrogen (Carlsbad, CA). Oligonucleotides were from MWG Biotech (High Point, NC) and Genscript (Piscataway, NJ). All other reagents were reagent grade or better and were purchased from Sigma (St. Louis, MO).

Strains and Growth Conditions. The *Escherichia coli* strain DH5 α (F-φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-) was used for all routine subcloning work. *Pichia pastoris* strains KM71 and X-33 from Invitrogen were used for heterologous expression of PfMDR1 isoforms. Yeast strain X-33 harboring pPICZc/3′Pfmdr16HB, KM71 harboring pPIC3.5/3′Pfmdr16HB, and KM71 harboring pPICZc/5′Pfmdr16HB or pPICZc/Pfmdr16HB were selected for growth on YPD medium + 100 mM zeocin, minimal glycerol medium lacking histidine, and minimal glycerol medium supplemented with 100 mM zeocin, respectively.

Design and Synthesis of the Synthetic (Yeast Optimized) WT pfmdr1 Gene. Design and synthesis of the yeast optimized WT *pfmdr1* gene followed procedures outlined previously (*14*) with a few modifications. Because of the very large size of *pfmdr1* and previous observations that suggest that 12 helix-2 NBD ABC transporters can be well expressed as half transporter cassettes (N- or C-terminal half 6 helix-1 NBD polypeptides; see ref *15*), we first constructed the yeast optimized gene as two cassettes (*pfmdr5′* and *pfmdr3′*). The first 2250 nucleotides of the *pfmdr1* gene sequence were obtained from GenBank (www.ncbi.nlm.ni-

h.gov) and translated. A total of 114 40-mers were constructed that encoded both strands of the theoretically optimized gene (*14*). Equal volumes of all 40-mers were combined (1.5 μM each), and the resultant mixture was diluted 25-fold in *Pfu*Ultra buffer supplemented with 0.5 mM each dNTP, 2 mM Mg^{2+}, and 1.25 U *Pfu*Ultra. The initial assembly PCR program was one denaturation step at 95 °C for 1 min, followed by 40 cycles of 95 °C (45 s), 52 °C (45 s), 72 °C (3 min), and finally a 10 min incubation at 72 °C. One microliter of this assembly solution was diluted 50-fold in similar buffer but with 1 μM of oligonucleotides #1 and #58 (the 5′- and 3′-flanking primers). The amplification PCR program was one denaturation step at 95 °C for 1 min, followed by 30 cycles of 95 °C (1 min), 60 °C (1 min), 72 °C (5 min), and a final 10 min incubation at 72 °C. After subcloning and propogation in *E. coli*, several PCR product clones were fully sequenced, and the one with the fewest errors was selected for further work. All spurious PCR errors were corrected using the multisite directed mutagenesis kit (Stratagene) and the completed gene confirmed by sequencing in both directions. Similar procedures were followed to synthesize the last 2115 nucleotides of the yeast optimized *pfmdr1* sequence (*pfmdr3′*), using the custom gene synthesis services of the Genscript corporation (Piscataway, NJ). Yeast expression vectors based on pPIC3.5 or pPICz from Invitrogen (see above) and containing either *pfmdr5′*, *pfmdr3′*, or the fused full length *pfmdr1*, an inducible alcohol oxidase promoter, and other convenient added features as described previously (*14*) were used to express WT and mutant (see below) PfMDR1 proteins.

Synthesis of Mutant pfmdr1 Genes. Three of the PfMDR1 mutations that have been proposed to play a role in antimalarial drug resistance are found in the C-terminal cassette (*pfmdr3′*), whereas two others are found in the N-terminal cassette. Three primers were designed to create the common S1034C, N1042D, and D1246Y mutations. The multisite directed mutagenesis kit (Stratagene) was used according to manufacturer's instructions. pPICZc/*pfmdr3′* was used as template with the following primers:

5′-CGCTGCACTTTGGGGATTC***T***GCCAATCGGCACAACTGTTC-3′,

5′-AATCGGCACAACTGTTCATC***G***ACTCGTTCGCGTACTGGTT-3′, and

5′-TGCGACTACAACCTTAGG***T***ACCTACGAAACCTCTTCTCAA-3′, respectively.

To create mutations in the 5′ cassette, found in PfMDR1 Dd2 (N86Y) or 7G8 (Y184F) isoforms, we used the following oligos:

5′-GGTTATCTTGAAGAACATG***T***ACTTGGGGGACGATATCAAC-3′, and

5′-GTCTTTCCTTGGGCTGT***T***CATCTGGTCCCTGATCAAGAAC-3′, respectively.

Yeast Transformations. Yeast were transformed with either the LiCl method using 50 μg of salmon sperm DNA as the carrier or via the *Pichia* transformation kit (for the pPICZc plasmids). Both methods used 3 μg of linearized target DNA. Transformants were plated on either minimal dextrose or YPD supplemented with 100 mM zeocin.

Isolation of Yeast Crude Membranes. Yeast cells were grown to midlog phase and induced with minimal methanol medium. Cells were harvested 18−24 h post induction, and crude cellular membranes were isolated via a glass bead protocol (*16*) and stored at −80 °C.

Purification of Yeast Plasma Membranes Harboring PfMDR1 Proteins. We followed the procedures in ref *16* with some modifications as in refs *17* and *18*. Freshly prepared crude membranes were diluted to 3.0 mg/mL in precipitation buffer (10 mM imidazole/1 mM $MgCl_2$/pH 5.20), adjusted to pH 5.20 with 100 mM HCl, and centrifuged at 7500*g* for 5 min. The supernatant was adjusted to pH 7.50 with 100 mM NaOH and centrifuged at 100,000*g* for 1 h at 4 °C. The resulting PM was resuspended in suspension buffer (10 mM imidazole/1 mM $MgCl_2$/pH 7.5), protein was quantified by amido black and densitometry (see below), and PM was aliquoted and stored at −80 °C.

Biotin and Polyhistidine Detection. SDS−PAGE gels (7.5%) were run for 100 min at 110 V, and protein was transferred onto polyvinylidene difluoride membranes at 40 mA for 16 h at 4 °C. For biotin detection, the membranes were washed once for 5 min with PBS-T (20 mM PO_4^{-3}/150 mM NaCl/pH 7.4), incubated for 1 h with 10% dried milk in PBS-T, washed again for 5 min in PBS-T, and then incubated in PBS-T supplemented with streptavidin-HRP following the manufacturer's instructions. For the poly histidine blots, the PentaHis detection kit from Qiagen was used according to manufacturer's instructions.

ATPase Activity Assays. The ATPase activity of purified PM fractions was measured using the colorimetric determination of orthophosphate released from ATP as described previously (*16*, *17*), but performed on a microliter scale in 96-well plates. Briefly, plates were set up on ice; assay buffer (180 mM NH_4Cl/100 mM Mes-Tris/10 mM $MgCl_2$/0.01% NaN_3, at various pH (see Results)) was added to each well followed by relevant drug solutions and finally membrane samples, to a total volume of 100 μL. The plate was shaken at 650 rpm on MixMate for 1 min and warmed to 37 °C using a water bath for exactly 1 min, and ATP was then added to all wells. After the plate was shaken (165 rpm) at 37 °C using a shaking incubator, stopping and stabilizing solutions were added at indicated times. (These are made weekly and stored at 4 °C.) Typically, 100 μL of the stopping/ascorbate mixture was added to each well followed 10 min later by 100 μL of stabilizing solution. Absorbance at 720 nm was read exactly 30 min after stabilizer was added, using a Victor 3V plate reader. Fresh solutions of 6% sodium ascorbate in 1 N HCl and assay buffer at various pH were made daily. Stocks solutions of ATP and drugs, and stock suspensions of PMs were thawed on ice, used once, and then discarded.

Negative control membranes from zeocin-resistant yeast transformed with empty vector (pPICzc) were included in all assays. Absorbance readings were converted to nmol Pi using a standard curve generated with K_2HPO_4 samples. Values for PM samples were then also scaled for total amount of protein according to densitometry results for silver stained gels as described in Results. Values for different PfMDR1 samples were normalized versus relative PfMDR1 content as described in Results. Replicates (at least three for each condition in each assay) were averaged, and the average control value under each assay condition was subtracted from the average for each PfMDR1 sample. All data shown are the results from multiple assays using at least two independent PM preparations for each PfMDR1 isoform, ±SD.

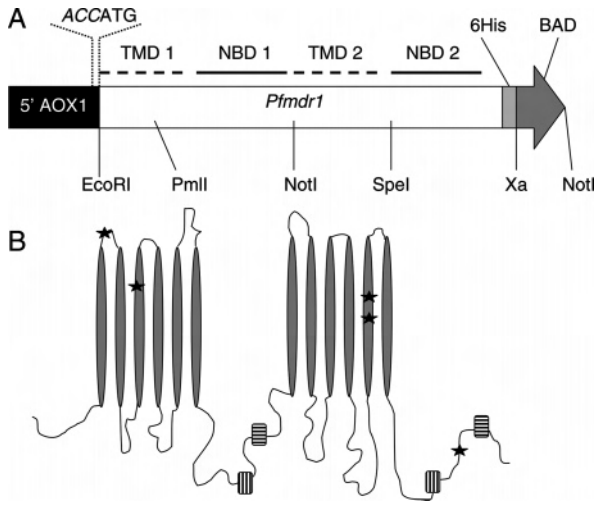


FIGURE 1: (A) Schematic of the yeast optimized PfMDR1 gene created for this work. The position of the alcohol oxidase promoter (AOX), an engineered Kozak sequence, restriction sites unique to the ORF, predicted 6 transmembrane helix domains (TMDs), nucleotide binding domains (NBDs), and fused hexa histidine (6His) as well as biotin acceptor domain (BAD) are indicated. (B) Cartoon of the predicted PfMDR1 secondary structure indicating helices (gray), loops (lines), and Walker A (vertical stripes)/Walker B (horizontal stripes) motifs within the NBD. The stars indicate the relative location of PfMDR1 point mutations previously associated with drug resistance (residues 84, 184, 1034, 1042, and 1246) found in the Dd2 and 7G8 isoforms vs 3D7 (see also Table 2).

When comparing the activity of the half transporter constructs to those of full length WT PfMDR1, data were normalized versus relative protein and relative hexa His label reactivity as above, and then also normalized versus relative number of NBD. Although activity of Pgp half transporters have been analyzed in a qualitative sense (*15*), to our knowledge, turnover quantification following this degree of densitometry-based normalization (see Results) has not previously been done.

RESULTS

We have previously overexpressed the *P. falciparum* membrane transporter PfCRT in *P. pastoris* and *S. cerevisiae* yeast (*14*). In those studies, it was readily apparent that the native cDNA did not express in yeast because of high AT content characteristic of *P. falciparum* genes, which manifests a large number of unpreferred codons, premature truncation signals, and other unfavorable features (*14*). Similarly, early attempts to reproducibly express native *pfmdr1* cDNA either failed or proved unreliable and at best led to vanishingly low levels of expression that could not be directly verified by western blot or other measurements (*13*). We therefore back translated the PfMDR1 amino acid sequence using a yeast preferred codon table and random codon seeding values as described (*14*). Upon inspecting numerous hypothetical genes for repeats and other potentially deleterious features, one gene was chosen and further tailored to remove poly A regions, insert convenient restrictions sites, and fuse C-terminally disposed biotin acceptor and hexa histidine tags (Figure 1). The final base and codon composition of this fully synthetic 4.59 kbp gene and comparison to average *P. falciparum* versus *P. pastoris* codon usage are shown in Table 1. The linear sequence is available in Supporting Information.

Our initial expression attempts focused on half transporter or cassette constructs as described in Materials and Methods. Upon successful, inducible, and approximately equal expression of yeast optimized 5′ and 3′ cassettes (see below), genes encoding the cassettes were fused together, and full length wild type (strain 3D7) PfMDR1 was successfully expressed to high levels (Figure 2A , lane 3) in inducible fashion (Figure 2C). The protein was found to be very well localized to the PM (Figure 2B, compare lanes 1−6). The initial assembly of the full length gene construct using introduced unique restriction sites created three additional alanine codons at the half transporter junction site (codon 749 in the primary amino acid sequence). These Ala codons did not affect expression levels, cellular localization, or measured ATPase activity (not shown, see below).

After optimizing the expression of the full length 3D7 isoform, point mutations were introduced as described in Materials and Methods to create genes encoding full length Dd2 and 7G8 PfMDR1 isoforms (see Table 2 and footnote). These full length proteins were also inducibly expressed to similar levels in *P. pastoris* (Figure 2A, lanes 4 and 5). We note that of the three isoforms, 7G8 PfMDR1 appears to express and insert into the PM to a higher extent (Figure 2A, compare lane 5 to lanes 3 and 4). We analyzed the kinetics of expression and PM insertion after MeOH induction (see Materials and Methods), and noted that the relative rate of PM integration for all isoforms is similar (not shown; representative membrane insertion kinetics shown in Figure 2C).

Expression of the full length PfMDR1 proteins in yeast was stable, as little obvious evidence of degradation was seen in hexa His gel analysis of purified PM fractions (e.g., Figure 2A), upon gel analysis of isolated cell fractions (e.g., Figure 2B) or upon analysis of expression kinetics (Figure 2C). No PfMDR1 was found in the cytosolic fraction 24 h post induction (Figure 2B, lane 1), and crude membrane fractions (Figure 2B, lane 3) or purified PM (lane 5) did not release PfMDR1 upon washing with high salt or chaotrope (e.g., 4 M NaCl wash, compare lane 3 vs 4 and 5 vs 6). However, we note that occasionally, biotin blots of full length PfMDR1 (e.g., Figure 3, discussed below) showed two minor protease−inhibitor sensitive bands that may represent minor degradation products produced during PM isolation.

A number of important ABC protein structure−function issues revolve around communication between the two homologous 6 helix TMD/1 NBD halves that are arranged in tandem fashion in full length ABCBs (Figure 1). We therefore also engineered expression vectors for the half transporters as previously described for human P-glycoprotein (Pgp) (*15*). Expression levels of all three constructs (5′ half transporter, 3′ half transporter, and full length) are comparable by either biotin or hexa His detection (Figure 3A and B respectively), and proceed via similar kinetics (not shown); however, expression and PM insertion of the N-terminally disposed half transporter (5′ encoded) is measureably higher than those of the C-terminal (3′ encoded) (Figure 3B; compare lane 7 vs 8). The full length Dd2 and 7G8 resistance-associated isoforms could also be stably expressed as half transporters, either individually or together (data not shown), similar to the case for the 3D7 isoform (Figure 3).

Table 1: Codon Usage and Base Composition for the Synthetic PfMDR1 Gene Used in This Work

amino acid	codon	*P. falciparum* usage (% codon used per amino acid)	*P. pastoris* usage (% per amino acid)	ncbi *pfmdr1* gene (exact number used)	synthetic 5′*pfmdr1* gene (exact number used)	synthetic 3′*pfmdr1* gene (exact number used)
Ala	GCA	43	26	30	6	1
	GCC	11	25	5	4	6
	GCG	4	5	0	5	11
	GCT	42	44	25	13	8
Arg	AGA	62	47	41	3	2
	AGG	15	17	0	7	3
	CGA	9	10	3	1	9
	CGC	2	6	0	2	7
	CGG	1	5	0	1	4
	CGT	12	16	7	6	5
Asn	AAC	15	50	16	90	57
	AAT	85	50	129	1	2
Asp	GAC	13	42	5	32	31
	GAT	87	58	75	7	11
Cys	TGC	14	35	1	7	5
	TGT	86	65	13	4	0
Stop	TAA	73	48	0	0	0
	TAG	11	30	0	0	0
	TGA	17	22	1	0	0
Gln	CAA	88	61	24	2	5
	CAG	12	39	4	12	9
Glu	GAA	87	59	65	11	10
	GAG	13	41	12	31	28
Gly	GGA	44	32	35	10	8
	GGC	4	14	0	6	5
	GGG	7	10	2	6	4
	GGT	44	44	31	17	17
His	CAC	17	44	3	6	7
	CAT	83	56	15	4	7
Ile	ATA	53	20	58	1	2
	ATC	7	31	6	71	55
	ATT	40	49	74	3	8
Leu	CTA	7	12	5	0	11
	CTC	2	8	4	13	18
	CTG	2	16	1	27	19
	CTT	11	17	10	4	7
	TTA	64	15	107	0	0
	TTG	13	32	10	34	5
Lys	AAA	82	48	119	1	2
	AAG	18	52	10	68	61
Met	ATG	100	100	34	16	19
Phe	TTC	18	43	11	32	50
	TTT	82	57	72	0	2
Pro	CCA	51	39	15	5	4
	CCC	10	16	113	2	3
	CCG	4	10	2	5	0
	CCT	35	35	8	3	7
Ser	AGC	6	9	57	7	9
	AGT	32	15	33	17	17
	TCA	27	19	45	11	7
	TCC	8	20	14	24	17
	TCG	4	8	2	11	12
	TCT	23	29	23	16	4
Thr	ACA	51	26	29	3	5
	ACC	12	23	8	24	12
	ACG	8	11	3	9	9
	ACT	28	40	25	4	3
Trp	TGG	100	100	6	5	2
Tyr	TAC	11	52	1	30	29
	TAT	89	48	63	2	3
Val	GTA	42	16	33	2	8
	GTC	6	22	2	18	11
	GTG	11	20	5	2	13
	GTT	41	41	31	13	5

To test whether the expressed proteins were functional, we analyzed ATPase activity versus time, [ATP], [Mg^{++}], pH, and several ATPase inhibitors. Using the plate-based assay described in Materials and Methods, activity exhibited inhibition at high [ATP] with an optimum near 5 mM (Figure 4A), was linear with time for at least 15 min (Figure 4B), was clearly Mg^{++} dependent (Figure 4C), had high activity at alkaline pH (Figure 4D), and had relatively low sensitivity

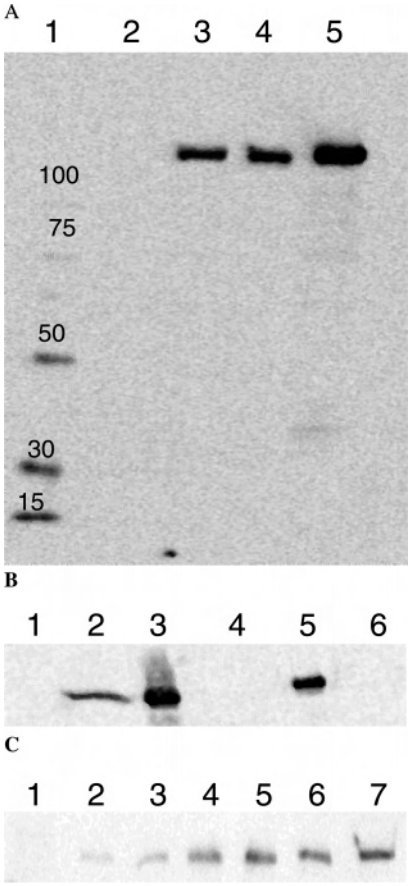


FIGURE 2: (A) Approximately equal expression of PfMDR1 isoform 3D7 (lane 3) Dd2 (lane 4), and 7G8 (lane 5) in purified *P. pastoris* PM as described in the text. Lane 1 is hexa His mole mass standards, and lane 2 is purified PM for similarly MeOH induced *P. pastoris* harboring empty pPICzc vector (Materials and Methods). The full length PfMDR1 migrates at 161.69 kDa (predicted mass = 171.81 kDa). Each lane harbors 15 μg of protein. (B) Analysis of relative PfMDR1 abundance in various cell fractions and assessment of membrane integration. Lane 1, cytosolic fraction; lane 2, 18000*g* pellet after cell rupture; lane 3, isolated crude membrane (CM) fraction; lane 4, 3.8 M NaCl wash of CM; lane 5, purified PM fraction; lane 6, 3.8 M NaCl wash of PM. Each lane harbors 40 μg of protein. Greater than 80% of expressed full length PfMDR1 was found in the crude membrane fraction (lane 3), and >60% of that was recovered in highly purified plasma membrane fractions (e.g., lane 5). (C) Time course of MeOH induction of 3D7 PfMDR1 in *P. pastoris*. Lane 1, 0 h; lane 2, 3 h; lane 3, 6 h; lane 4, 9 h; lane 5, 12 h; lane 6, 18 h; lane 7, 24 h. Each lane harbors 20 μg of protein.

Table 2: PfMDR1 Isoforms[a]

allele	N86Y	Y184F	S1034C	N1042D	D1246Y
3D7 (wild type)	N	Y	S	N	D
Dd2	Y	Y	S	N	D
7G8	N	F	C	D	Y

[a] The amino acid substitutions at codon positons 86, 184, 1034, 1042, and 1246 (denoted by stars in Figure 1B) for the different PfMDR1 isoforms analyzed in this study.

to vanadate (Figure 4E) but high sensitivity to concanamycin (Figure 4F). Interestingly, the Dd2 PfMDR1 isoform was considerably more active under basal conditions and showed a higher pH optimum relative to 3D7 (Figure 4D triangles/dashed line vs squares/solid line), yet the other drug resistance-associated isoform (7G8; circles/dotted line) was

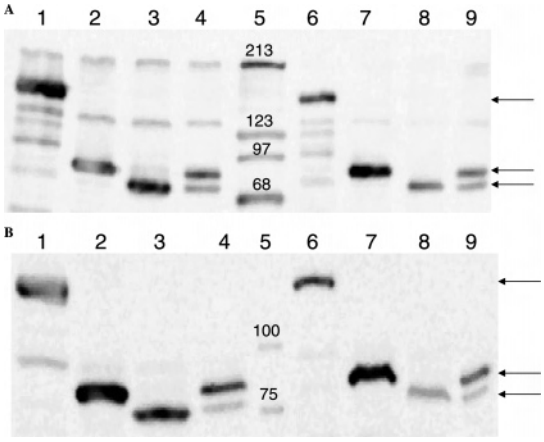


FIGURE 3: (A) Biotin detection blot of WT full length (lanes 1 and 6) vs N- (lanes 2 and 7) and C-terminal (lanes 3 and 8) half transporters. Each lane harbors 10 μg of protein. The full length PfMDR1 migrates at 161.69 kDa (first arrow right-hand side; predicted mass = 171.81 kDa), N-terminal polypeptide migrates at 87.44 kDa (second arrow; predicted mass = 94.69 kDa), and the C-terminal migrates at 77.03 kDa (third arrow; predicted mass = 86.94 kDa). Lanes 1−4 are crude yeast membrane fractions, lane 5 is biotinylated MW standards, and lanes 6−9 are purified PM fractions. Lanes 1 and 6, membranes expressing full length PfMDR1; lanes 2 and 7, N-terminal half transporter; lanes 3 and 8, C-terminal; lanes 4 and 9, both N-terminal and C-terminal half transporters coexpressed. (B) Parallel hexa His blot of the same samples as in A, except each lane harbors 40 μg of protein.

less active relative to 3D7. Although a range of characteristics can be noted in the literature for ABC proteins, in general, this ATPase activity is similar to that of other 12 helix ABCB transporters. However, we note that at high [ATP], the ATPase activity of PfMDR1 is particularly robust relative to human or mouse Pgp, yeast PDR5, and other ABCB involved in drug resistance phenomena, and is instead more similar to the basal activity previously measured for prokaryotic ABC transporters (see Discussion). Using densitometry to rigorously quantify PfMDR1 in the yeast PM (Figure 5), we calculated apparent K_m and V_{max} for wild type PfMDR1 of 2.14 mM and 62.9 μmol Pi released/mg PfMDR1/min, respectively, at pH 7.50 and 10 mM Mg^{++} (Table 3). Other eukaryotic 12 helix ABC transporters have been reported to exhibit K_m values for basal ATPase activity that range from 0.5 to 2.5 mM and V_{max} of 0.5−5 μmol/mg/min. However, relatively few eukaryotic ABC V_{max} values have previously been quantified using integral native membrane preparations and are instead more often calculated using purified detergent extracted enzyme that could conceivably have lost some level of activity. Also, unlike PfMDR1 (see below), other ABC proteins exhibit up to 20-fold stimulation in the presence of various drugs (see Discussion). Thus, basal ATPase activity of heterologously expressed PfMDR1 is commensurate with the higher end of previously measured, optimized (e.g., drug stimulated) activity for various ABC transporters but to our knowledge represents the highest level of basal activity yet recorded for a 12 helix ABCB protein.

A hallmark feature of most ABC transporters involved in drug resistance phenomena is that drugs to which they confer resistance either stimulate or inhibit their ATPase activity. As summarized elsewhere (*19*), reported effects are usually stimulatory, mild (2−5-fold), or quite significant (10−20-fold), and are often, but not always, further modulated by VPL and other chemo-reversal agents. However, the range

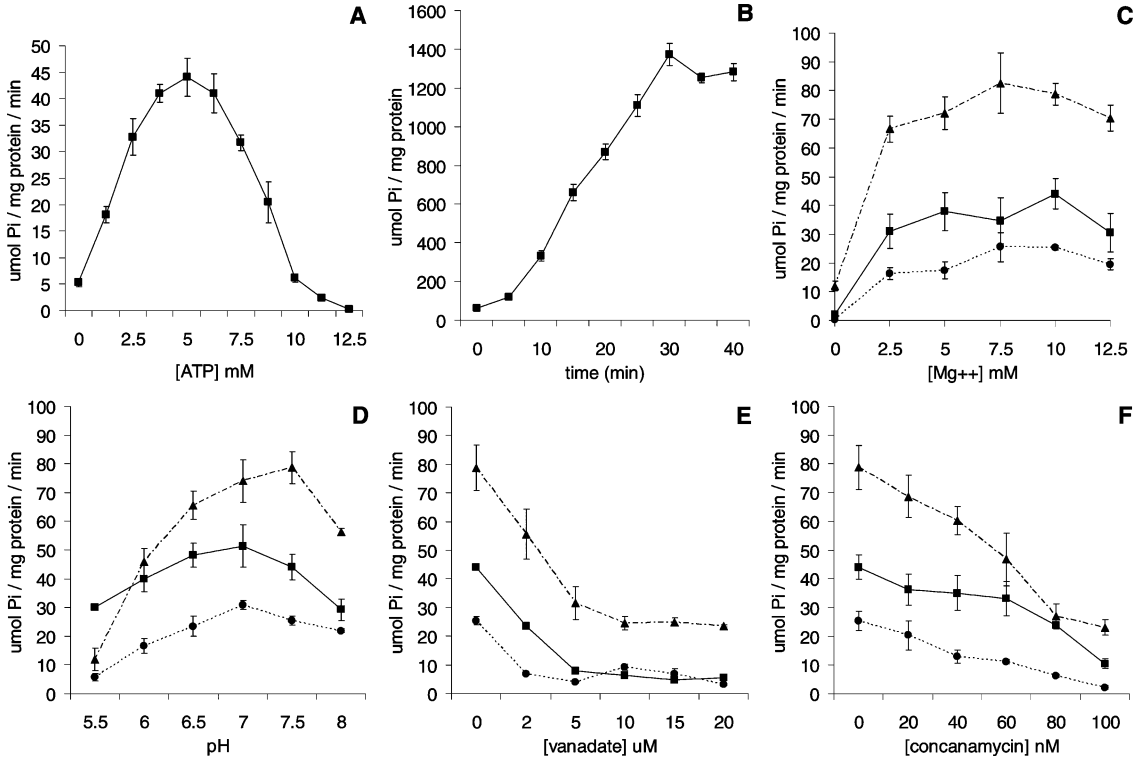


FIGURE 4: ATPase activity of recombinant PfMDR1 isoforms. PfMDR1 ATPase plate assay vs [ATP] (A) and time (B) for WT (3D7) PfMDR1. The results for Dd2 and 7G8 isoforms (not shown) are similar in shape but have different amplitude as described in the following Figures. Also summarized is Mg^{++} dependence (C), pH dependence (D), vanadate sensitivity (E), and concanamycin sensitivity (F) for 3D7 (■, solid lines, all panels), Dd2 (▲, dashed lines) and 7G8 (●, dotted lines) PfMDR1 isoforms.

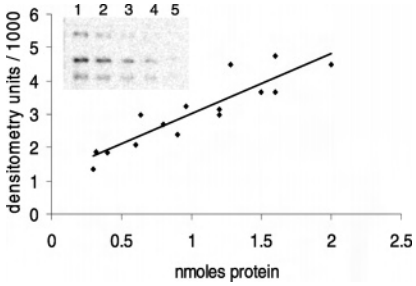


FIGURE 5: Densitometry standardization of PfMDR1 membrane content. Hexa His molecular mass standards (Bio Rad) were serially diluted (inset, lanes 1−5) to yield varible nanograms of protein per lane (150, 120, 90, 60, 30; 120, 96, 72, 48, 24; and 100, 80, 60, 40, 20 for 100 kDa (inset, top band); 75 kDa (middle); and 50 kDa (bottom) standards, respectively). After staining and quantitative densitometry using procedures identical to those for PfMDR1-containing membranes and conversion to moles, we plotted density vs nmol hexa His tagged protein (♦). We used a linear fit to these data ($y = 1802.8x + 1201.7$, $R^2 = 0.81$) and the predicted molecular mass of 171.81 kDa to determine nmol PfMDR1 per mg PM. Dividing this by the amount of PM protein loaded on various gels and averaging across several gels, we find that at 24 h induction PfMDR1 isoforms 3D7, Dd2, and 7G8 constitute 1.19%, 1.46%, and 3.07% of PM protein, respectively (see also Figure 2). These data also allow us to quantitatively compare turnover results across multiple PM samples and multiple PfMDR1 isoforms (or half transporters).

of drug concentrations used in such assays varies widely and is often nonphysiologic. In the case of PfMDR1 (which resides within the digestive vacuolar (DV) membrane), the definition of the physiologically relevant drug concentration that should be examined is somewhat ambiguous. For example, *in vitro* IC_{50} data suggest that 10−20 nM CQ is physiologically relevant for CQS strains (e.g., strain 3D7), whereas 100−200 nM is relevant for CQR (e.g., those

Table 3: Kinetic Parameters and Inhibitor Sensitivities[a]

	3D7	Dd2	7G8
pH optimum	7.0	7.5	7.0
V_{max} (μmol Pi/mg/min)	62.9	109.9	42.7
K_m (mM)	2.14	2.00	3.42
vanadate IC_{50} (μM)	2.25	4.00	1.25
concanamycin IC_{50} (nM)	82.6	67.4	42.9

[a] pH optima, V_{max}, K_m, and sensitivity to vanadate and concanamycin for the different PfMDR1 isoforms analyzed in this study. IC_{50} values were calculated using a sigmoidal fit to the data shown in Figure 4.

expressing Dd2 and 7G8 PfMDR1 alleles or overexpressing the 3D7 allele (see Discussion)). Parasite cytosolic concentrations for CQ and other quinolines are predicted to be near growth medium concentrations (*20*); therefore, if drugs interact with PfMDR1 via the cytosolically disposed face (the face harboring ABCB NBDs), then concentrations near IC_{50} would be physiologically relevant with respect to possible PfMDR1 interactions. However, the DV membrane maintains a high ΔpH, which acts to strongly concentrate weak base antimalarial drugs within the DV. If the drug is effectively diprotic (e.g., CQ), then this effect goes as the square of the net pH gradient (the pH scale is logarithmic), whereas if the drug is effectively monoprotic (e.g., MQ and QN), then it is linearly related to the pH scale (*20*).

Therefore, we analyzed PfMDR1 ATPase activity with or without MQ, QN, and CQ in either the absence (Figure 6) or the presence (Figure 7) of VPL and over two concentration ranges that reflect reasonable anticipated equilibrium cytosolic (lower range, left-hand side of each panel in Figures 6 and 7) and DV (higher range, right-hand side of each panel in Figures 6 and 7) concentrations. (Data for both ranges are consolidated in Figures 6 and 7.) CQ, MQ, and QN were

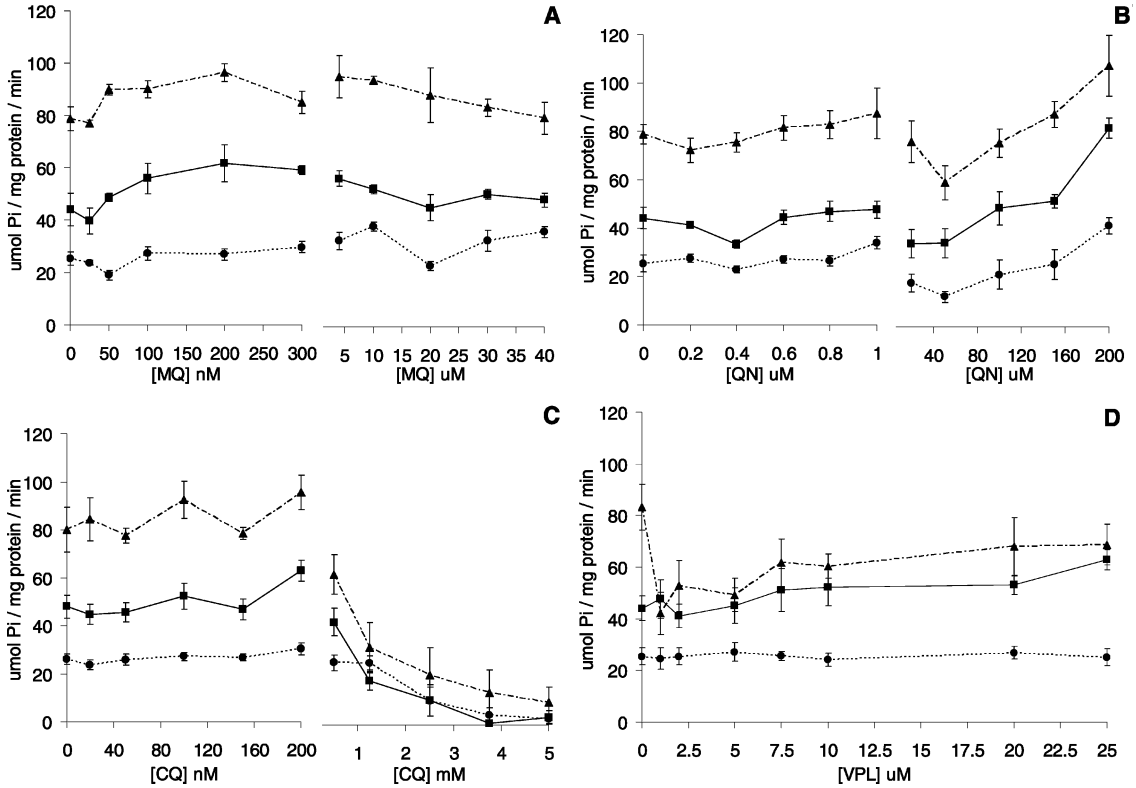


FIGURE 6: PfMDR1 ATPase activity under standard conditions ([ATP] = 5 mM; [Mg^{++}] = 10 mM; time = 15 min) vs MQ (A), QN (B), CQ (C), and VPL (D) for 3D7 (■, solid lines) Dd2 (▲, dashed lines), and 7G8 (●, dotted lines) isoforms. In this Figure and Figure 7, we consolidate data over two physiologically relevant concentration ranges that reflect expected drug concentrations either in the parasite cytosol (left-hand side; 0.2 × IC_{50} to 5 × IC_{50}) or the digestive vacuole (right-hand side; the cytosolic range × pH gradient fold concentration expected for monoprotic (MQ and QN) or diprotic (CQ) quinolines (*20*)).

chosen for this initial analysis because on the basis of data with field isolates (*5*) and *P. falciparum* transfectants (*8*, *9*), the hypotheses that have been offered to date include that mutation and/or overexpression of PfMDR1 confers the highest degree of resistance to MQ and QN but no appreciable resistance to the related drug CQ. (However, in the Discussion section, we note some disagreement in the literature with respect to this last point.) Whether or not the mutation of PfMDR1 to Dd2 or 7G8 isoforms confers higher levels of MQ or QN resistance compared to 3D7 is controversial but has been a popular hypothesis.

Drugs to which ABC transporters confer resistance are expected to stimulate ATPase activity; thus, surprisingly, QN did not stimulate PfMDR1 over the range of concentrations that corresponds to expected cytosolic (0−400 nM Figure 6A and B). Over a wide range of MQ concentrations that spans both expected cytosolic and digestive vacuolar (0.05−5.0 μM), a mild (30−40%) but reproducible stimulatory effect was measured for 3D7 and Dd2 PfMDR1 isoforms (Figure 6A, squares and triangles, respectively), in multiple independent purified PM preparations (Materials and Methods). The effect was proportionally greatest for 3D7 PfMDR1. Interestingly, the 7G8 isoform (Figure 6A, circles) was not appreciably stimulated at similar [MQ].

A significant QN stimulation was found for 3D7 and Dd2 isoforms (Figure 6B, squares and triangles, respectively) but only at very high dosages that correspond to the upper limit of what is expected within the DV- for QN-resistant *P. falciparum*. Again the effect was proportionally greatest for 3D7 and significantly reduced for the 7G8 isoform. At 200 μM QN, the stimulatory effect was nearly 60% (1.6-fold) and 100% (2-fold) for the Dd2 (triangles, Figure 6B) and 3D7 (squares) isoforms, respectively, and, similar to the case for MQ, noticeably less for the 7G8 isoform (circles).

Interestingly, the largest effects on ATPase activity were seen for CQ (Figure 4C), to which PfMDR1 is not believed to confer resistance (*1*, *6*−*9*). A trend in mild stimulation of ATPase activity was seen for the 3D7 and Dd2 isoforms at the higher range of anticipated cytosolic concentrations (Figure 6C, left-hand side top two traces) that was of marginal significance. However, CQ then very strongly inhibited all isoforms at anticipated DV concentrations (Figure 6C, right-hand side). Net stimulation was largest for the 3D7 isoform and again was not apparent for the 7G8 isoform; however, in relative terms, the 7G8 isoform was inhibited to a similar extent at high [CQ].

In some cases, VPL alone and/or non lethal doses of VPL along with drugs to which the ABC transporter confers resistance further stimulates ATPase activity of the transporter. Physiologic (nontoxic) dosages of VPL (1−2 μM) had a significant effect on Dd2 PfMDR1 ATPase activity but not on 3D7 and 7G8, which appeared insensitive over this range (Figure 6D). The Dd2 isoform was inhibited by approximately 2-fold at 1 μM VPL, and 40−50% stimulation from this value was then seen at progressively higher (toxic) concentrations (Figure 6D, triangles). Again, the 7G8 isoform was much less influenced by the drug; in this case, virtually no effect was measured for this isoform (Figure 6D, circles) across a wide range of [VPL].

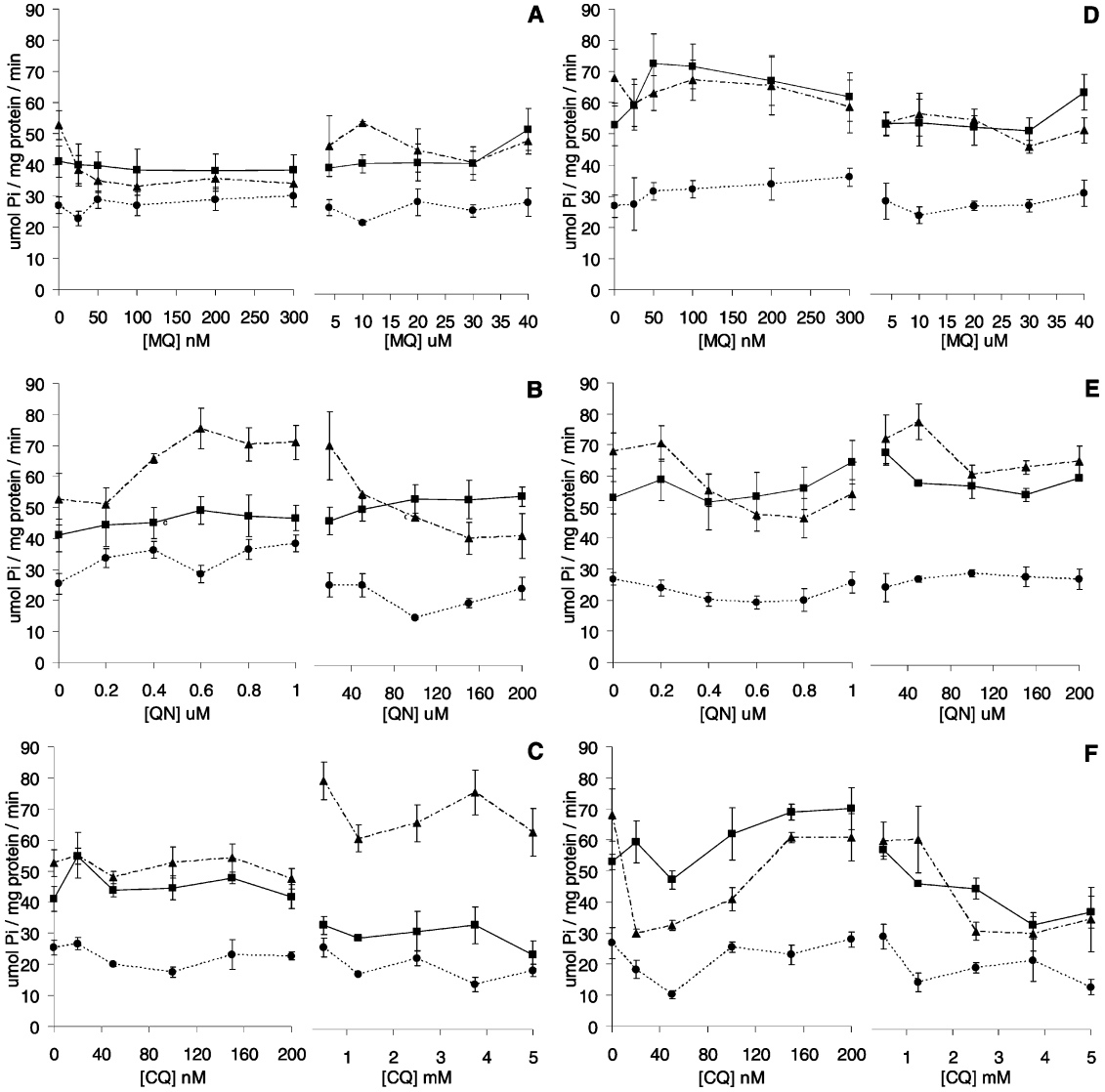


FIGURE 7: PfMDR1 ATPase activity vs MQ (A and D), QN (B and E), and CQ (C and F) for 3D7 (■, solid lines), Dd2 (▲, dashed lines), and 7G8 (●, dotted lines) isoforms in the presence of either 2 μM (A, B, and C) or 20 μM (D, E, and F) VPL. Note that we consolidate data over two physiologically relevant quinoline concentration ranges that reflect either expected parasite cytosol (left-hand side) or digestive vacuole (right-hand side) concentrations.

In the case of the MQ and QN stimulatory effects noted above, 1−2 μM VPL did not further stimulate but in fact negated or shifted the concentration dependence for MQ- or QN-induced stimulation, respectively (Figure 7A and B). Similar to the effect of VPL alone, the effects were noticeably larger for the Dd2 isoform, wherein 2 μM VPL in combination with anticipated DV concentrations of MQ (e.g., 200 nM) actually inhibited ATPase activity more than VPL alone (compare Figure 6A to Figure 7A triangles). VPL abrogated stimulation at 200 μM QN (Figure 7B vs Figure 6B) but interestingly shifted QN stimulation for Dd2 to lower [QN] (Figure 7B, triangles). At 2 μM concentration, VPL did not fully negate the ATPase inhibition seen at high doses of CQ for the 3D7 isoform (Figure 6C, squares) but did so for the Dd2 isoform (Figure 7C, triangles). In sum, in these examples, chemosensitizing doses of VPL along with quinoline appear to have primarily opposing effects on ATPase activity, relative to the effects seen in the presence of the same dose of quinoline antimalarial alone.

Finally, we also examined ATPase activity of the 3D7 half transporter constructs (Materials and Methods and ref *15*) expressed either alone (N term and C term; see *x*-axis) or together (coex) and compared this to the behavior of the full length 3D7 isoform (FL) (Figures 8 and 9). As expected (*15*), ATPase activity of N term and C term was lower than that of FL (Figure 8A and B), even after normalization to number of NBDs (Materials and Methods), yet they exhibited similar [ATP] dependency (Figure 8A, open circles and open triangles vs squares, respectively). The two half transporters expressed together (coex; Figure 8A, solid diamonds) did not show maximum ATPase activity until higher [ATP], relative to FL 3D7 (squares). Interestingly, the pH dependency for ATP hydrolysis was lost for the half transporters yet was steeper for coex (Figure 8B). Unlike the case for Pgp (*15*), wherein N term activity was found to be noticeably higher than C term activity, and the basal activity of coex Pgp (both Pgp N term and C term polypeptides expressed together) was approximately 70% that of the FL Pgp,

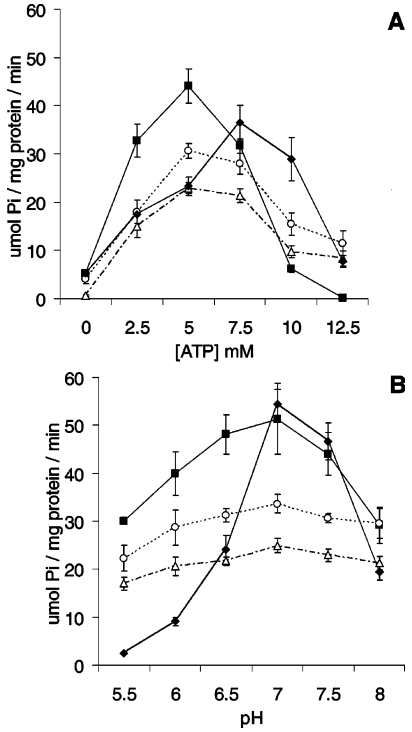


FIGURE 8: ATPase of individual WT half transporters vs WT coex vs WT full length. (A) Dependency on [ATP]. (B) pH dependency. Full length, (■, solid lines); N-terminal, (△, dashed line); C-terminal, (○, dotted line); coexpressed halves, (♦, thatched line).

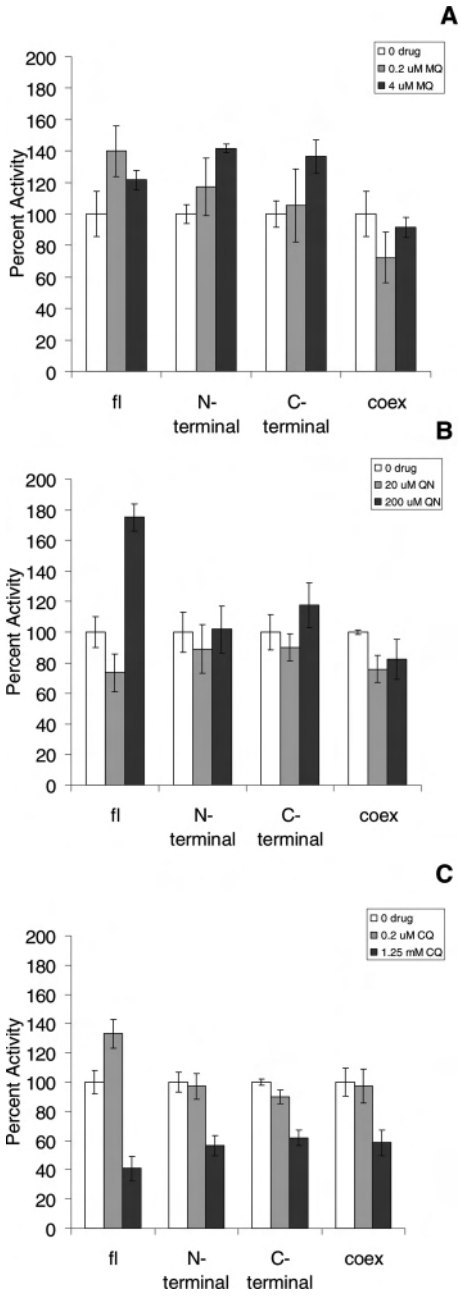


FIGURE 9: ATPase of individual WT half transporters (N-terminal and C-terminal) vs WT coex (both half transporters expressed together in the same cell) vs WT full length (fl) in the presence of no drug (open bars) vs cytosolic (gray bars) or DV (black bars) concentrations of MQ (A), QN (B), and CQ (C).

PfMDR1 N term was found to have similar activity relative to C term, and at optimum pH, coex PfMDR activity was 100% that of the FL PfMDR1. On the basis of these data, it is likely that communication between the two halves (which is also believed to be important with regard to drug stimulation effects) is quite different for PfMDR1 relative to the well studied Pgp.

Similar to the drug effects noted for Pgp half transporters (*15*), QN and CQ drug stimulatory effects observed for the FL PfMDR1 were lost when the protein was expressed as N term or C term forms (Figure 9B and C). However, mild MQ stimulation, but at higher dose (corresponding to predicted DV concentrations), was preserved for the N- and C-terminal half transporters (Figure 9A, gray vs black bars) as were inhibitory effects seen at high dose CQ (Figure 9C, solid bars).

DISCUSSION

Reproducible high level overexpression of large polytopic integral plasma or vacuolar membrane proteins is frequently difficult and for *P. falciparum* proteins has only been accomplished after *de novo* synthesis of optimized genes (*14*). Yet, molecular analysis of several of these (PfCRT, PfNHE1, and PfMDR1) is crucial for further understanding the molecular basis of antimalarial drug resistance. In this study, we have used methods similar to those reported previously for the much smaller PfCRT protein (*14*) to create synthetic yeast optimized versions of *pfmdr1* genes and now report reproducible, high level overexpression of WT (strain 3D7) PfMDR1 and two common isoforms (Dd2 and 7G8) that are expressed in common CQR laboratory strains of *P. falciparum*. The proteins are well expressed in *P. pastoris*, well localized to the yeast PM in either half transporter or full length forms, and are efficiently biotinylated when an *E. coli* transcarboxylase biotin acceptor domain is fused to the C-terminus. After optimizing a plate-based assay, we used purified yeast PM fractions to perform an initial characterization of PfMDR1 isoform ATPase activities versus key antimalarial drugs.

The definition of the molecular mechanism behind any ABCB transporter-mediated drug resistance phenomenon remains elusive. In the particular case of PfMDR1 versus antimalarial drug resistance, there is some disagreement and controversy in the relevant literature. First, importantly, any antimalarial resistance mediated *in vivo* by mutated or

alternately expressed PfMDR1 appears to be dependent upon the simultaneous expression of CQR-conferring mutant PfCRT (*1*). That is, mutation and/or increased expression of PfMDR1 likely does not promote appreciable drug resistance in and of itself, as shown recently for strain GC03 parasites that express wild type PfCRT (*8*). The nature of this PfCRT/PfMDR1 interaction, whether it is direct or indirect and so forth, is not known but obviously merits additional study using heterologous expression and purification methods. Second, the relative importance of PfMDR1 overexpression versus mutation (as well as overexpression of WT vs overexpression of mutant PfMDR1 isoforms) in contributing to antimalarial drug resistance is currently a topic of debate. Notwithstanding these two complexities, the data in this article do help to further define an emerging model for the role of PfMDR1 in antimalarial drug resistance.

From prokaryotes to humans, there are multiple examples wherein either overexpression or mutation of either 6 helix or 12 helix ABC proteins contributes to pleiotropic resistance phenomena (resistance to antibiotics, heavy metals, antiparasitic drugs, anticancer drugs, etc.). In reviewing all of this, it is difficult and perhaps dangerous to attempt to draw too many general conclusions. However, there are important comparisons that are relevant for interpreting these data for PfMDR1. PfMDR1 is a 12 helix 2 NBD member of the ABCB subfamily that is believed to confer resistance to hydrophobic weakly basic compounds. These drugs are in some respects structurally reminiscent of cancer chemotherapeutics, to which overexpression of the human ABCB protein MDR1 (P-glycoprotein; Pgp) confers resistance. A similar parallel could be drawn using the yeast ABCB protein PDR5, which is known to be similar to Pgp in many respects (*21*).

Reports of how Pgp or PDR5 ATPase activities are altered by the drugs to which these proteins confer resistance vary (*15*, *19*, *21*−*27*), but with a few exceptions, they are stimulatory and typically at least 2−3-fold and occasionally even as high as 10−20-fold. Notably, PfMDR1 differs from these related resistance conferring ABCB proteins in that the drug to which it is currently believed to confer the highest level of resistance (MQ) stimulates ATPase activity by at most only 30−40%. Also, again in contrast to Pgp and PDR5, the MQ chemoreversal agent VPL does not further stimulate within the relevant concentration range. This suggests, but does not prove, that relative to Pgp and PDR5 versus drugs to which they confer resistance, MQ interacts more weakly with PfMDR1, and VPL may interact in a different fashion altogether. This would be consistent with PfMDR1 playing only a small role in conferring resistance to MQ and other quinoline-based antimalarials, as has been recently suggested by several laboratories based on QTL or transfection results (*4*, *8*, *9*). ATPase stimulation is somewhat greater at very high levels of QN, but even in this case, it is below what has typically been measured for Pgp or PDR5. Overall then, drug stimulation of PfMDR1 ATPase activity is unlike that for any previously characterized drug resistance associated ABCB protein and is instead curiously much more consistent with recent results for Cdr1p, a member of the architecturally distinct ABCG subfamily that is involved in antifungal drug resistance (*28*). We note that PfMDR1 may confer mildly altered sensitivity to other antimalarial compounds outside the quinoline class (e.g., reactive endoperoxides (*9*)), but this is currently controversial, and if it is indeed correct, the effect is very mild.

The CQ profile, differences in this profile for 3D7 versus Dd2 isoforms, and the effects of VPL on these profiles are intriguing. The biphasic (mild stimulatory followed by strongly inhibitory) profile observed across the wide range of [CQ] inspected is unusual but is again somewhat reminiscent of Pgp, which has been observed to exhibit biphasic profiles for several compounds to which it mediates resistance (*19*). But because the prevailing hypothesis is that PfMDR1 does not mediate resistance to CQ, these data are surprising. The nearly complete inhibition of PfMDR1 by high DV concentrations of CQ could be quite important for interpreting complex patterns of active versus passive drug transport proposed to be linked to the CQR phenotype (*29*, *30*).

In the case of Pgp, although exceptions can be found, VPL typically further stimulates ATPase activity when combined with weak base chemotherapeutics that stimulate. In the present case, 2 μM VPL reverses the stimulatory effect seen at 200 nm MQ and 200 μM QN as well as the mild CQ-based stimulation and completely reverses the CQ inhibition seen for Dd2 PfMDR1 but not 3D7. These effects could be due in part to the fact that unlike Pgp, PfMDR1 does not appear to be simulated by VPL alone to any appreciable extent. A weak interaction with VPL is consistent with the recent conclusions of Cooper et al. (*31*) that link VPL chemoreversal of quinoline drug resistance in *P. falciparum* to PfCRT mutations and not to the mutation or altered expression of PfMDR1. Conversely, Kirk and colleagues (*32*) have recently suggested that PfMDR1 mutations influence the intrinsic antiplasmodial activity of VPL. (The compound is toxic to *P. falciparum* above 2 μM.) Although the effects are mild, we do note differences in how 7G8 versus Dd2 versus 3D7 isoforms respond to VPL. ATPase activity of the 7G8 isoform is not affected by VPL alone, and data in ref *32* suggest that mutations found in the 7G8 isoform confer increased sensitivity to VPL. Thus, stimulation of PfMDR1 ATPase by toxic levels of VPL (Figure 5D, right side, top traces) may be linked to decreased sensitivity to VPL. This might be consistent with 3D7 and Dd2 PfMDR1 acting to concentrate VPL within the DV, away from targets elsewhere in the cell.

How the other ATPase effects we measure correspond to patterns of drug resistance believed to be partially mediated by PfMDR1 *in vivo* is less clear. As mentioned, for *P. falciparum*, low levels of resistance that may be mediated by PfMDR1 require the presence of mutated PfCRT, and the relative importance of PfMDR1 overexpression versus mutation in contributing to this resistance is currently a topic of debate. Recent field studies (*5*) suggest that for isolates harboring CQR-conferring *Pfcrt* mutations, gene duplication of wild type (3D7) *pfmdr1* is associated with increased resistance to MQ and QN but does not further influence the level of CQR. *In vitro*, some increased resistance to MQ and QN was seen for strains expressing the N86Y (Dd2) mutant isoform (*5*), but no *in vivo* correlation was observed. Relatedly, recent gene disruption studies using a strain exhibiting *pfmdr1* gene duplication presumably due to CQ selection (*33*) have shown the converse, namely, that decreased expression of PfMDR1 confers increased susceptibility to MQ and QN.

In light of these observations, interestingly, the basal ATPase activity we measure is conspicuously higher for Dd2 and 3D7 isoforms versus 7G8, with Dd2 being the highest. Although allele specific overexpression trends have not been examined in any detail to our knowledge, on the basis of this result, we predict that selection for overexpression of 7G8 PfMDR1 would be less common than overexpression of wildtype or Dd2 isoforms in drug resistant *P. falciparum*. Furthermore, our data suggest that if the net level of PfMDR1 ATPase activity is the relevant factor in conferring resistance, overexpression of Dd2 PfMDR1 under CQ selection pressure would be more efficient than overexpression of WT. Meaning, in the presence of CQS IC_{50} levels of CQ (the clinically relevant level of CQ selection pressure), two copies of Dd2 PfMDR1 would provide similar increased PfMDR1 ATPase activity relative to four copies of 3D7 (nearly 10 copies of 7G8 would be required to obtain the same level of PfMDR1 ATPase activity). Interestingly, we note that Dd2 CQR parasites show elevated cytosolic pH (*20* and refs therein) relative to 3D7, which would act to further stimulate PfMDR1 (Dd2 isoform of PfMDR1) in these strains. That is, if overexpression of PfMDR1 is indeed a small but important contribution to antimalarial drug resistance and because ATPase activity is fundamental to how this ABCB protein confers drug resistance (as is the case for all other ABCB), we predict that higher levels of overexpression of WT isoforms (meaning >2 copies) will be found to be more common than that for Dd2. We also predict that overexpression of 7G8 or 7G8-like isoforms yet to be discovered will be less well correlated with drug resistance (relative to 3D7 and Dd2) because reasonable increases in copy number will not impart similar levels of drug stimulated PfMDR1 ATPase activity. We also note that in further predicting the relative effects of these PfMDR1 isoforms, precise quantification of cytosolic [ATP] and pH for the malarial parasite will be crucial. Only a handful of measurements have been reported, but available data suggest (*34*) that due to anaerobic metabolism, ATP concentrations are lower than those for other eukaryotes, perhaps close to 1−2 mM.

Because ABCB proteins are believed to translocate drug in some fashion from the NBD-disposed side of the membrane (in this case, cytosolic) to the opposite side (in this case, the DV interior) and because the DV is believed to be the primary site of action for MQ, QN, and other quinolines, how PfMDR1 concentrating more drug within the DV leads to resistance has always been a lingering mystery. If the DV is indeed where the primary drug target (ferriprotoporphyrin IX heme) resides, as is generally accepted to be the case (*20*), this would act to encourage drug−target interactions, which is the converse of what is typically promoted by any drug resistance mechanism. As one possibility, we have previously observed that quinoline drugs actually act to prematurely precipitate heme dimers from solution (*35*). These curious nucleation phenomena, which occur at different rates as the drug is varied from vastly substoichiometric to high molar excess, would lower drug target availability. If pH, volume (concentration of heme), and ionic strength were manipulated in certain ways (as indeed seems to be the case for drug resistant malaria (*36*)), synergistic (and quinoline drug specific) heme aggregation would occur.

That is, in all other examples of ABCB-mediated drug resistance of which we are aware, the proteins function to lower the accumulation of drug on the NBD side of the membrane (e.g., the cytosol for a drug resistant tumor cell) because the NBD side is disposed toward the drug target (e.g., cytosolic tubulins in the case of Pgp mediated resistance to vinca alkaloids, colchicines, etc.). Alternatively, resistance-conferring ABC proteins expressed in vacuolar membranes (again with NBD disposed to the cytosol) act to concentrate drug into the vacuole in order to again sequester the drug away from the drug target, which is cytosolically or nuclearly localized. In this case, because PfMDR1 is expressed in the DV membrane, the simple prediction is that PfMDR1 would act to concentrate drugs *at* their site of action (inside the DV where the heme target lies), not *away*. Higher levels of PfMDR1 (as in drug resistant field isolates overexpressing *pfmdr1*) or the drug stimulation of PfMDR1 ATPase shown here would be predicted to concentrate even *more* drug at the site of action (not less). This is again paradoxical, unless this has an unexpected effect on target availability as described in ref *35*. Alternatively, although the concept has not been as extensively explored as has the drug interaction concept, it is also true that drug resistance-conferring members of the ABC transporter family have been observed to mediate movement of ions under various conditions (*17*, *37*). This possibility for PfMDR1 merits additional scrutiny because the pH gradient across the DV membrane is very large and directly or indirectly controls the accumulation of CQ, MQ, QN, and other drugs within the DV as well as the biomineralization of the heme drug target. Even subtle effects on this parameter and others closely linked to it (e.g., DV volume and other ion activities (*36*)), which via this model could then be influenced by drug effects on PfMDR1 ATPase activity, would contribute to resistance in interesting and drug specific ways (*35*).

Along with a better understanding of the nature of quinoline−heme interactions (*35*, *36*), obviously many additional molecular questions remain. The perfected heterologous expression described in this article will be extremely useful in addressing these. For example, it will be interesting in future work to dissect the ATPase effects observed in naturally occurring (e.g., Dd2 and 7G8) versus artificial (*8*) PfMDR1 isoforms versus the wild type. Some of these amino acid substitutions lie within homologous regions that are believed to be involved in drug binding for other ABCB proteins (i.e., residues 1034 and 1042 mutated in 7G8 PfMDR1, which lie within predicted helix 11). Also, how PfCRT and PfMDR1 might interact to further modify either these PfMDR1 properties or PfCRT properties (*14*) will be important to examine.

In conclusion, our data showing higher basal ATPase and shifted pH optimum for the Dd2 PfMDR1 isoform, mild stimulation of PfMDR1 ATPase by physiologically relevant doses of MQ and QN, equal or even slightly higher stimulation for wild type versus other isoforms, and VPL reversal (not simulation) of these effects are in general consistent with the evolving picture presented in refs *5*, *8*, and *9*. Assuming these drug effects on PfMDR1 are relevant to the drug resistance mechanism, overexpression of wild type PfMDR1 will confer many of the same effects, regardless of mutation, to Dd2 or 7G8 isoforms, and in some cases, higher levels of wild type would be predicted to be

more effective than increased expression of some CQR-associated isoforms (i.e., 7G8).

ACKNOWLEDGMENT

MQ was a kind gift of Dr. M. Ferdig (Notre Dame). We thank the GenScript corporation for help with gene synthesis, and the Lombardi Cancer Center and Northwestern University for DNA sequencing support. We also thank our laboratory colleagues for experimental help, particularly M. Paguio, M. Cabrera, and B. Vaccarro. P.D.R. acknowledges helpful conversations with Drs. M. Ferdig and R. Cooper.

SUPPORTING INFORMATION AVAILABLE

The full length sequence of the synthetic 3D7 *pfmdr1* gene used in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Fidock, D. A., Nomura, T., Talley, A. K., Cooper, R. A., Dzekunov, S. M., Ferdig, M. T., Ursos, L. M. B., Sidhu, A. B. S., Naude, B., Deitsch, K. W., Su, X. Z., Wootton, J. C., Roepe, P. D., and Wellems, T. E. (2000) Mutations in the P-falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance, *Mol. Cell 6*, 861−871.
2. Sidhu, A. B., Verdier-Pinard, D., and Fidock, D. A. (2002) Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by pfcrt mutations, *Science 298*, 210 − 213.
3. Plowe, C. V. (2005) Antimalarial drug resistance in Africa: strategies for monitoring and deterrence, *Curr. Top. Microbiol. Immunol. 295*, 55−79.
4. Ferdig, M. T., Cooper, R. A., Mu, J. B., Deng, B. B., Joy, D. A., Su, X. Z., and Wellems, T. E. (2004) Dissecting the loci of low-level quinine resistance in malaria parasites, *Mol. Microbiol. 52*, 985−997.
5. Price, R. N., Uhlemann, A. C., Brockman, A., McGready, R., Ashley, E., Phaipun, L., Patel, R., Laing, K., Looareesuwan, S., White, N. J., Nosten, F., and Krishna, S. (2004) Mefloquine resistance in *Plasmodium falciparum* and increased pfmdr1 gene copy number, *Lancet 364*, 438−447.
6. Wellems, T. E., Panton, L. J., Gluzman, I. Y., do Rosario, V. E., Gwadz, R. W., Walker-Jonah, A., and Krogstad, D. J. (1990) Chloroquine resistance not linked to mdr-like genes in a *Plasmodium falciparum* cross, *Nature 345*, 253−255.
7. Wellems, T. E., Walker-Jonah, A., and Panton, L. J. (1990) Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7, *Proc. Natl. Acad. Sci. U.S.A. 88*, 3382−3386.
8. Reed, M. B., Saliba, K. J., Caruana, S. R., Kirk, K., and Cowman, A. F. (2000) Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*, *Nature 403*, 906−909.
9. Sidhu, A. B., Valderramos, S. G., and Fidock, D. A. (2005) pfmdr1 Mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*, *Mol. Microbiol. 57*, 913−926.
10. Duraisingh, M. T., and Cowman, A. F. (2005) Contribution of the pfmdr1 gene to antimalarial drug-resistance, *Acta Trop. 94*, 181−190.
11. Myrick, A., Munasinghe, A., Patankar, S., and Wirth, D. F. (2003) Mapping of the *Plasmodium falciparum* multidrug resistance gene 5′-upstream region, and evidence of induction of transcript levels by antimalarial drugs in chloroquine sensitive parasites, *Mol. Microbiol. 49*, 671−683.
12. Ruetz, S., Delling, U., Brault, M., Schurr, E., and Gros, P. (1996) The pfmdr1 gene of *Plasmodium falciparum* confers cellular resistance to antimalarial drugs in yeast cells, *Proc. Natl. Acad. Sci. U.S.A. 93*, 9942−9947.
13. Ruetz, S., Delling, U., Brault, M., Schurr, E., and Gros, P. (1999) The pfmdr1 gene of *Plasmodium falciparum* confers cellular resistance to antimalarial drugs in yeast cells, *Proc. Natl. Acad. Sci. U.S.A. 96*, 1810.
14. Zhang, H., Howard, E. M., and Roepe, P. D. (2002) Analysis of the antimalarial drug resistance protein PfCRT expressed in yeast, *J. Biol. Chem. 277*, 49767−49775.
15. Loo, T. W., and Clarke, D. M. (1994) Reconstitution of drug-stimulated ATPase activity following co-expression of each half of human P-glycoprotein as separate polypeptides, *J. Biol. Chem. 269*, 7750−7755.
16. Dufour, J. P., and Goffeau, A. (1980) Molecular and kinetic properties of the purified plasma membrane ATPase of the yeast *Schizosaccharomyces pombe*, *Eur. J. Biochem. 105*, 145−154.
17. Howard, E. M., and Roepe, P. D. (2003) Purified human MDR 1 modulates membrane potential in reconstituted proteoliposomes, *Biochemistry 42*, 3544−3555.
18. Fritz, F., Howard, E. M., Hoffman, M. M., and Roepe, P. D. (1999) Evidence for altered ion transport in *Saccharomyces cerevisiae* overexpressing human MDR 1 protein, *Biochemistry 38*, 4214−4226.
19. Scarborough, G. A. (1995) Drug-stimulated ATPase activity of the human P-glycoprotein, *J. Bioenerg. Biomembr. 27*, 37−41.
20. Ursos, L. M., and Roepe, P. D. (2002) Chloroquine resistance in the malarial parasite, *Plasmodium falciparum*, *Med. Res. Rev. 22*, 465−491.
21. Decottignies, A., Kolaczkowski, M., Balzi, E., and Goffeau, A. (1994) Solubilization and characterization of the overexpressed PDR5 multidrug resistance nucleotide triphosphatase of yeast, *J. Biol. Chem. 269*, 12797−12803.
22. Urbatsch, I. L., al-Shawi, M. K., and Senior, A. E. (1994) Characterization of the ATPase activity of purified Chinese hamster P-glycoprotein, *Biochemistry 33*, 7069−7076.
23. Regev, R., Assaraf, Y. G., and Eytan, G. D. (1999) Membrane fluidization by ether, other anesthetics, and certain agents abolishes P-glycoprotein ATPase activity and modulates efflux from multidrug-resistant cells, *Eur. J. Biochem. 259*, 18−24.
24. Loo, T. W., and Clarke, D. M. (1995) Rapid purification of human P-glycoprotein mutants expressed transiently in HEK 293 cells by nickel-chelate chromatography and characterization of their drug-stimulated ATPase activities, *J. Biol. Chem. 270*, 21449−21452.
25. Sharom, F. J., Yu, X., Chu, J. W., and Doige, C. A. (1995) Characterization of the ATPase activity of P-glycoprotein from multidrug-resistant Chinese hamster ovary cells, *Biochem. J. 308*, 381−390.
26. Rogers, B., Decottignies, A., Kolaczkowski, M., Carvajal, E., Balzi, E., and Goffeau, A. (2001) The pleitropic drug ABC transporters from *Saccharomyces cerevisiae*, *J. Mol. Microbiol. Biotechnol. 3*, 207−214.
27. Decottignies, A., Grant, A. M., Nichols, J. W., de Wet, H., McIntosh, D. B., and Goffeau, A. (1998) ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p, *J. Biol. Chem. 273*, 12612−12622.
28. Shukla, S., Rai, V., Banerjee, D., and Prasad, R. (2006) Characterization of Cdr1p, a major multidrug efflux protein of *Candida albicans*: purified protein is amenable to intrinsic fluorescence analysis, *Biochemistry 45*, 2425−2435.
29. Rohrbach, P., Sanchez, C. P., Hayton, K., Friedrich, O., Patel, J., Sidhu, A. B., Ferdig, M. T., Fidock, D. A., and Lanzer, M. (2006) Genetic linkage of pfmdr1 with food vacuolar solute import in *Plasmodium falciparum*, *EMBO J. 25*, 3000−3011.
30. Sanchez, C. P., Stein, W., and Lanzer, M. (2003) Trans stimulation provides evidence for a drug efflux carrier as the mechanism of chloroquine resistance in *Plasmodium falciparum*, *Biochemistry 42*, 9383−9394.
31. Cooper, R. A., Lane, K. D., Deng, B., Mu, J., Patel, J. J., Wellems, T. E., Su, X., and Ferdig, M. T. (2007) Mutations in transmembrane domains 1, 4 and 9 of the *Plasmodium falciparum* chloroquine resistance transporter alter susceptibility to chloroquine, quinine and quinidine, *Mol. Microbiol. 63*, 270−82.
32. Hayward, R., Saliba, K. J., and Kirk, K. (2005) Mutations in pfmdr1 modulate the sensitivity of *Plasmodium falciparum* to the intrinsic antiplasmodial activity of verapamil, *Antimicrob. Agents Chemother. 49*, 840−842.
33. Sidhu, A. B., Uhlemann, A. C., Valderramos, S. G., Valderramos, J. C., Krishna, S., and Fidock, D. A. (2006) Decreasing pfmdr1 copy number in plasmodium falciparum malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin, *J. Infect. Dis. 194*, 528−535.

34. Kanaani, J., and Ginsburg, H. (1989) Metabolic interconnection between the human malarial parasite *Plasmodium falciparum* and its host erythrocyte. Regulation of ATP levels by means of an adenylate translocator and adenylate kinase, *J. Biol. Chem. 264*, 3194−3199.
35. Ursos, L. M., DuBay, K. F., and Roepe, P. D. (2001) Antimalarial drugs influence the pH dependent solubility of heme via apparent nucleation phenomena, *Mol. Biochem. Parasitol. 112*, 11−17.
36. Gligorijevic, B., Bennett, T., McAllister, R., Urbach, J. S., and Roepe, P. D. (2006) Spinning disk confocal microscopy of live, intraerythrocytic malarial parasites. 2. Altered vacuolar volume-regulation in drug resistant malaria, *Biochemistry 45*, 12411−12423.
37. Venter, H., Shilling, R. A., Velamakanni, S., Balakrishnan, L., and Van Veen, H. W. (2003) An ABC transporter with a secondary-active multidrug translocator domain, *Nature 426*, 866−870.

BI7002026

Available online at www.sciencedirect.com
ScienceDirect
Molecular & Biochemical Parasitology 157 (2008) 107–111
MOLECULAR & BIOCHEMICAL PARASITOLOGY

Short communication

A single S1034C mutation confers altered drug sensitivity to PfMDR1 ATPase activity that is characteristic of the 7G8 isoform

Jacqueline K. Lekostaj, Linda E. Amoah, Paul D. Roepe *

Department of Chemistry, Department of Biochemistry, Cellular & Molecular Biology, Tumor Biology Program, Lombardi Cancer Center, and Center for Infectious Disease, Georgetown University, 37th and O Streets, Washington, DC 20057, USA

Received 14 June 2007; received in revised form 26 September 2007; accepted 28 September 2007
Available online 5 October 2007

Abstract

The mechanism behind how PfMDR1 may contribute to antimalarial drug resistance is unclear. Transfection studies suggest that PfMDR1 mutations may make small contributions to drug sensitivity in a strain-dependent fashion, whereas field data link over expression (not necessarily mutation) of the gene with clinical drug treatment failure. This study dissects the contribution of individual mutations of PfMDR1 that contribute to the unique behavior of the 7G8 PfMDR1 isoform. A single mutation in putative TM 11 (S1034C) is found to abolish drug stimulation of PfMDR1 ATPase activity.

Keywords: Malaria; Drug; Vacuole; PfMDR1; ATPase

PfMDR1 is a member of the ATP-binding cassette (ABC) superfamily, many members of which (e.g. human P-glycoprotein; HuMDR1) have been implicated in drug resistance phenomena. PfMDR1 is composed of two homologous cassettes, each containing six putative transmembrane (TM) helices and one nucleotide-binding domain (NBD). Similar to HuMDR1, PfMDR1 has been proposed to confer multidrug resistance. A number of drug resistance-conferring ABC proteins have been proposed to function as drug transporters, using the energy from ATP hydrolysis to catalyze drug efflux from a cell or cellular compartment containing the relevant drug target, thereby promoting drug resistance. ATP-dependent small molecule or ion pumps in general do not hydrolyze ATP well in the absence of transported substrate. Thus, although basal ATPase activity in the absence of drugs remains to be understood, support for ABC drug transport models includes stimulation of ABC ATPase activity by the drugs to which the ABC transporter confers resistance. For example, stimulation of HuMDR1 ATPase is 3–10-fold for a number of drugs to which it confers resistance [1,2], and also up to 10-fold for compounds that reverse the resistance phenotype (e.g. verapamil [VPL] see [1]). Calculated drug effects on K_m span two orders of magnitude, and different patterns of significant K_m vs. V_{max} alterations have been noted [1]. Although strict correlations between the magnitude of ATPase stimulation vs. ability of the ABC transporter to confer resistance are typically not found, in general, a higher degree of ATPase stimulation by drugs to which the ABC transporter confers the highest degree of resistance is a frequent observation (e.g. [1,2] and references within).

Many questions surround the role of PfMDR1 in antimalarial drug resistance phenomena. The presumed importance of PfMDR1 in drug resistance has been significantly diminished by genetic studies that culminated in the recent discovery that *Pfcrt* mutations are the main molecular determinant of chloroquine resistance (CQR) for *Plasmodium falciparum* [4–6]. It has therefore subsequently been suggested that PfMDR1 modulates the level of CQR and resistance to other quinoline-based antimalarials in a PfCRT-dependent manner. Another hypothesis is that PfMDR1 mutations could represent fitness adaptations in CQR *P. falciparum* harboring mutated PfCRT. In terms of mechanism, the protein has been localized to the membrane of the parasite digestive vacuole (DV; [3]), with NBDs disposed towards the cytoplasm. Therefore, if the above drug pumping model is applicable, PfMDR1 would paradoxically act to translocate drugs into the DV, which is believed to be the principle site of action for most quinoline antimalarials. How concentrating

* Corresponding author. Tel.: +1 202 687 7300; fax: +1 202 687 7186.
E-mail address: roepep@georgetown.edu (P.D. Roepe).

0166-6851/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2007.09.008

drugs at the site of action would promote resistance is a puzzle. However, for non-quinoline drugs (such as artemisinin) with multiple unknown targets, some of which may reside in the cytosol, sequestration in the DV could act to promote resistance. If PfMDR1 recognizes and alters the diffusion of a wide variety of substrates (some of which have targets within the DV and others without), it may help to explain why increased resistance to one compound can be accompanied by hypersensitivity to another.

Transfection studies suggest that PfMDR1 mutations may make small contributions to sensitivity vs. quinine (QN), mefloquine (MQ), halofantrine (HF) and artemisinin (ART), but in a strain-dependent fashion [7,8]. However, field data link over expression (not necessarily mutation) of the gene with clinical drug treatment failure [9]. Thus the relative importance of PfMDR1 mutation vs. overexpression in adding to resistance profiles is currently debated. Laboratory experiments with field isolates commonly report mutations at five codons: N86Y and Y184F in the N-terminal cassette, and S1034C, N1042D and D1246Y in the C-terminal cassette. Using allelic exchange, the Cowman group [7] reported that introduction of the three C-terminal mutations into a chloroquine sensitive (CQS) strain conferred resistance to QN and mildly increased susceptibility to MQ, HF and ART; the MQ and HF effects were even more apparent in a strain bearing only the 1246Y mutation. However, effects of the individual mutations on drug-stimulated PfMDR1 ATPase activity are not known. Reversion of the same three mutations back to wildtype in a strain 7G8 background resulted in the opposite phenotype (MQ, HF, and ART resistance and QN sensitivity). These results were later confirmed in additional transfection studies by Sidhu et al. [8], which also suggested the 1042D mutation was particularly influential.

Taking all of the above together, it is commonly expected that if these PfMDR1 mutations do indeed promote additional degrees of resistance to particular drugs (e.g. QN, MQ), then effects of these drugs on the ATPase activity of the PfMDR1 mutants should be significant, and/or differ vs. effects for wild type PfMDR1. If this is the case, defining the specific contributions of individual mutations would assist identification of drug interaction sites. However, all previous work with PfMDR1 has until now been done with parasite isolates or transfectants, systems greatly complicated by the presence of other proteins involved in drug resistance (e.g. PfCRT) and/or other changes due to antimalarial drug selection pressure. Moreover, isolating parasite fractions within which PfMDR1 ATPase activity is easily separable from other ATPases is extremely difficult, if not impossible.

Thus, we have recently reported the successful heterologous overexpression of several PfMDR1 isoforms found in either CQS or CQR parasites [10], which now greatly facilitates molecular dissection of PfMDR1, including quantification of drug effects on ATPase activity, which as mentioned is a hallmark of drug resistance-conferring ABC transporters. While all overexpressed isoforms were active as measured by their ability to hydrolyze ATP [10], there were significant differences among them. In particular, the PfMDR1 isoform expressed in CQR strain Dd2 exhibited higher ATPase activity relative to that

Table 1
Summary of PfMDR1 mutant kinetic parameters

	3D7	Dd2	1034C	1042D	1246Y	1034C/1042D	1034C/1246Y	1042D/1246Y	TM (triple)	7G8
Basal ATPase (μmol Pi/(mg min)$^{-1}$	44.04 ± 4.72	78.73 ± 8.88	27.17 ± 3.33	70.08 ± 5.49	92.69 ± 7.87	69.59 ± 8.67	56.94 ± 5.69	45.34 ± 5.24	14.07 ± 1.32	25.38 ± 4.23
ATPase with 2 μM VPL	41.06 ± 4.46	52.69 ± 9.78	27.09 ± 0.86	61.56 ± 3.52	98.73 ± 9.86	78.05 ± 10.71	63.19 ± 7.20	34.79 ± 1.43	11.80 ± 1.54	25.38 ± 3.29
[ATP] optimum (mM)	5.0	5.0	5.0	5.0	7.5	7.5	2.5	7.5	7.5	5.0
pH optimum	7.0	7.5	7.0	7.5	7.0	7.0	7.0	7.0	7.0	7.0
V_{max} (μmol Pi/(mg min)$^{-1}$	62.9	109.9	46.9	70.9	109.9	93.5	59.5	73.0	49.3	42.7
K_m (mM)	2.14	2.00	5.53	0.369	0.879	1.82	0.226	2.78	8.15	3.42
Vanadate IC_{50} (μM)	2.25	4.00	4.36	4.55	4.36	7.00	3.65	4.29	N/A	1.25

Kinetic parameters and inhibitor sensitivities of PfMDR1 isoforms. Basal ATPase activity ±VPL (rows 1, 2) are measured under optimum conditions (temp, [ATP], pH) in the absence of antimalarial drugs, as described previously [8] and numbers shown are average of six measurements (see methods) ±S.D. For row 3, activity was tested at [ATP] = 2.5, 5.0, 7.5 and 10.0 mM; for row 4 activity was tested at pH 6.0–8.0 in 0.5 unit increments, and the [ATP] and pH at which highest activity was measured are then listed. For other rows, values were computed after curve fitting the relevant plotted variables as described in detail elsewhere [8]. VPL is verapamil, N/A denotes not analysed, as the measured activity in the presence of vanadate was too low to yield a statistically reliable calculation.

expressed in CQS 3D7, while activity of the CQR strain 7G8 isoform was by far the lowest of the three. Also, in contrast to Dd2 and 3D7 isoforms, ATPase activity of 7G8 PfMDR1 was largely unaffected by quinoline antimalarial drugs, except at very high [CQ]. Since Dd2 PfMDR1 possesses a single amino acid change relative to wildtype (N86Y) some changes in activity relative to wild type can be assigned to this substitution. However, 7G8 PfMDR1 contains four substitutions relative to wild type: Y184F, S1034C, N1042D, D1246Y. In order to investigate which of these mutations is responsible for unusual 7G8 isoform ATPase activity (e.g. low V_{max}, high K_m, conspicuous loss of QN and MQ stimulation), we have created yeast strains expressing PfMDR1 harboring various combinations of these mutations. This is the first step in identifying regions of the PfMDR1 molecule that may interact with specific drugs.

Initial data indicated that a mutant containing the three C-terminal substitutions (called "triple mutant" or TM by Reed et al. [7]) behaved similarly relative to 7G8 PfMDR1 (see below and also [7]). We therefore focused attention on the effects of 1034C, 1042D and 1246Y substitutions, both alone and in various possible combinations. Using recently published methods [10] we constructed three yeast strains expressing PfMDR1 with each single mutation as well as three strains expressing PfMDR1 with each possible double mutation (Table 1). We then purified plasma membrane fractions from each strain (Fig. 1) and quantified PfMDR1 ATPase activity as described [10]. All mutants tested had an optimum pH near 7.0 or 7.5 and all but one showed optimal activity near 5.0 or 7.5 mM ATP (Table 1). In terms of basal activity, the S1034C mutant closely approximates 7G8, whereas N1042D is more similar to Dd2, and D1246Y exhibits the highest ATPase activity of any mutant yet tested (with a V_{max} identical to that of Dd2). However, interestingly, K_m for S1034C is conspicuously higher relative to 7G8. That is, no single C-terminal substitution recapitulates both V_{max} and K_m seen for the 7G8 isoform. Two or more mutations must therefore act in concert to produce an enzyme with 7G8 kinetic characteristics.

Interestingly, the 1034/1042 and 1034/1046 double mutants both show elevated V_{max} and lower K_m relative to S1034C. The 1042/1246 double mutant is similar to the wildtype 3D7 isoform, and showed higher K_m relative to the single site 1042 and 1246 mutants. Thus the lower V_{max} and higher K_m relative to wild type that is seen for TM and 7G8 is not seen in any of the double mutants; all three C-terminal substitutions are required for this behavior (Table 1). The remaining mutation that converts TM to 7G8 (Y184F) produces a small effect that then mildly raises V_{max} and lowers K_m but, importantly, does not alter drug response (see below), consistent with previous suggestions from analysis of transfectants [7].

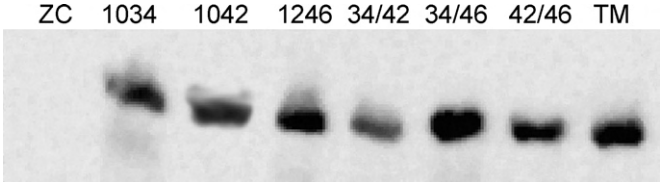


Fig. 1. Representative polyHis blot showing approximately equal inducible expression of new PfMDR1 mutants in the constructed yeast strains. Expression levels are similar to those of 3D7, Dd2 and 7G8 isoforms reported previously [8]. "ZC" indicates negative control carrying empty zeocin selectable vector. Yeast were grown under standard conditions, induced with methanol for ~20 h and plasma membranes purified by acid precipitation as described [10]. Protein was quantified by amido black and an equivalent amount of membrane protein was loaded into wells of a 7.5% polyacrylamide gel, which was run at 110 V for 100 min and subsequently transferred to a PVDF membrane overnight at 40 mA. The blot was developed using the PentaHis detection kit from Qiagen according to manufacturer's instructions. After averaging densitometry from two such plots, we calculate that for each mutant, PfMDR1 constitutes 3.63, 2.45, 2.71, 1.94, 2.85, 2.05 and 2.46% of total membrane protein, respectively.

We have previously reported [10] that the ATPase activity of wild type (3D7) PfMDR1 is mildly stimulated over a wide range of estimated cytoplasmic and digestive vacuolar concentrations of MQ (0–300 nM and 5–60 μM, respectively). Amongst the C-terminal mutants now tested (Fig. 2A), the N1042D and D1246Y single mutants as well as the 1034C/1042D double mutant (not shown) are similarly stimulated by MQ. In contrast, remarkably, the S1034C single mutant showed very little response upon addition of any concentration of MQ, very similar to the behavior of TM and 7G8. Other double mutants (1034/1246, 1042/1246) showed intermediate behavior (not shown, see caption).

A significant QN stimulatory effect was previously seen for wild type and Dd2 isoforms, but only at very high concentrations that correspond to the upper range of what we calculate to be expected within the DV [10]. None of our newly created C-terminal mutants display this stimulation, and indeed some even seem to be inhibited by high amounts of QN (Fig. 2B). However, D1246Y was stimulated to a similar extent by QN, but at much lower concentrations of QN.

Considering the fact that CQ is generally not believed to interact with PfMDR1 [7,8], it was surprising that we earlier found CQ had the greatest effect on ATPase activity: high DV-compatible concentrations of CQ severely inhibit both 3D7 and 7G8 isoforms. Under similar conditions, all C-terminal mutants exhibit some degree of inhibition, but only S1034C and TM are rendered almost completely inactive (Fig. 2C). In sum, of all mutants tested, only S1034C exhibited a nearly identical "drug profile" relative to 7G8 PfMDR1. Also, interestingly, S1034C and 7G8 are the only two isoforms that were not affected at all by the chemosensitizer verapamil (VPL) (Table 1).

Some parallels can be drawn between these PfMDR1 mutation sites and corresponding positions in the human MDR1 sequence. Topologically, the N86Y mutation is found in the DV-disposed loop between the first and second predicted TM domains. Similar to our previous results with Dd2 [10], deletions in this region of HuMDR1 lead to altered verapamil sensitivity [11]. The 1034 and 1042 mutations lie in the middle of predicted TM helix 11, which has been hypothesized to be part of a drug-binding site in HuMDR1 [12–14]. TM11 has also been implicated in the release of drug during ATP hydrolysis [15]. Thus our finding that the S1034C substitution has the most significant effect on PfMDR1 ATPase drug stimulation is consistent with previous drug interaction domain analyses for other ABC proteins. The D1246Y mutation is situated within the C-terminal NBD. While there are no direct comparisons to HuMDR1 currently in the literature, it seems likely that any mutation within the NBD is liable to affect ATP binding or hydrolysis. How

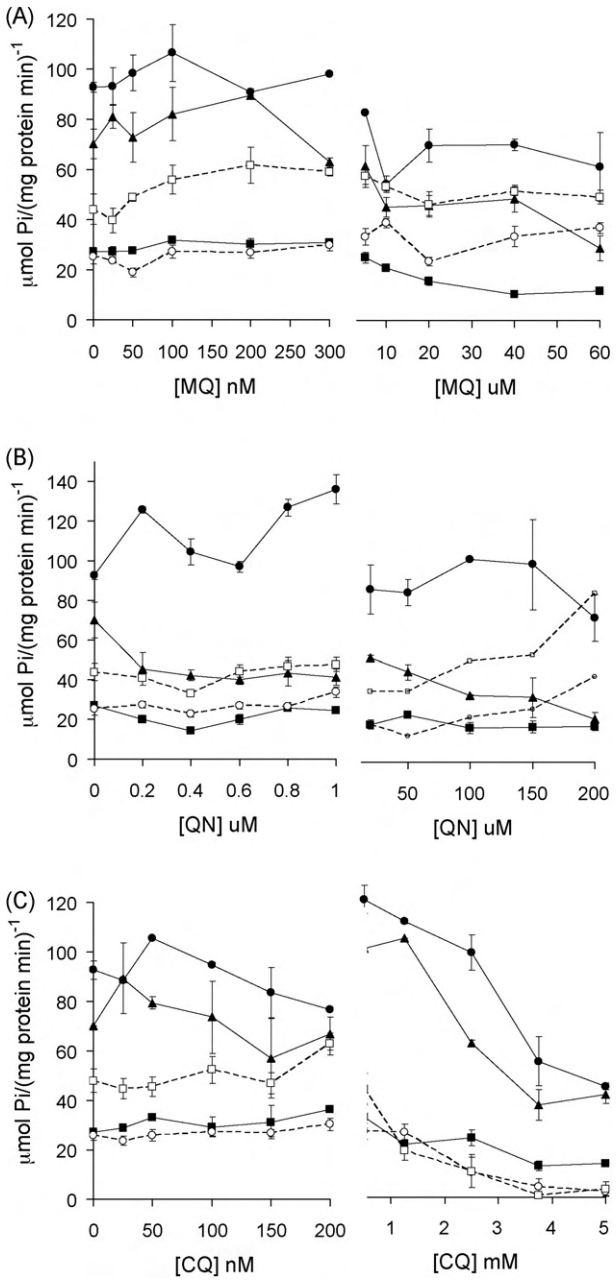


Fig. 2. ATPase activity of C-terminal mutants in the presence of varying amounts of MQ (A), QN (B) and CQ (C). Activity for 3D7, Dd2 and 7G8 is also reproduced in Ref. [10]. The ATPase activity of purified PM fractions was measured using the colorimetric determination of orthophosphate released from ATP as described in detail previously [10]. Briefly, plates were set up on ice: assay buffer pH 7.5 (180 mM NH_4Cl/100 mM Mes-Tris/10 mM $MgCl_2$/0.01% NaN_3) was added to each well followed by relevant drug solutions, and finally membrane samples, to a total volume of 100 μL. The plate was shaken, warmed to 37 °C, and ATP was then added. After shaking at 37 °C for an additional 15 min, the reaction was stopped, stabilized 10 min later, and absorbance at 720 nm read after 30 min. Results shown are the average (±S.D.) for at least two independent membrane preparations, each done at least in triplicate, and values are normalized to PfMDR1 content via densitometry as described in detail elsewhere [10]. Single mutants, solid lines, closed symbols: S1034C, closed squares; N1042D, closed triangles; D1246Y, closed circles. Strain isoforms, dashed lines, open symbols: 3D7, open squares; 7G8, open circles. Double mutant data are omitted for clarity but are available from the authors upon request: 1034/1042 ATPase activity is very similar to that of D1246Y when plotted vs. these concentrations of MQ, QN or CQ, whereas 1034/1246 and 1042/1246 show behavior that lies between that of D1246Y vs. 7G8 and S1034C. Note behavior for D1246Y is reminiscent of that seen for isoform Dd2 [10] indicating that single site mutations at two widely separate positions (1246 and 86, respectively) are capable of approximately doubling V_{max}.

this domain then interacts with others predicted to interact with drug (e.g. TM 11) to further modify V_{max} and K_m remains to be determined, but ongoing crystallographic analyses of other ABC proteins (e.g. [16]) will be helpful in this regard.

Since mutations in PfCRT originated in at least four independent foci worldwide [17], PfMDR1 mutations may have arisen in similar fashion, against a backdrop of selective drug pressure and fitness adaptation in response to different PfCRT mutations. In evolutionary terms, a number of scenarios are possible since it is unclear which quinoline drugs (if any) selected for Dd2 and 7G8 PfMDR. But based on our inspection of V_{max}, K_m, and drug stimulation of ATPase for all the mutants described herein, and assuming quinoline exposure was the penultimate driving force for PfMDR1 mutation, we speculate that the 7G8 strain first acquired the S1034C mutation in order to bias against drug effects on the enzyme, followed by the other substitutions, which merely act to "fine tune" V_{max} and K_m. Regardless the order, the final consequence is an enzyme that has reduced catalytic efficiency and that is insensitive to quinoline-based antimalarial drugs, both of which would seem to be advantageous based on conventional drug transport models for MDR proteins and the DV localization of both PfMDR1 and the quinoline target (see above). That is, both features would presumably act synergistically to lower quinoline drug accumulation into the DV via an ABC transporter with cytosolically disposed NBD. However, importantly, if we compare the behavior of Dd2, 7G8 (both CQR strains) and 3D7 (CQS) PfMDR1 isoforms [10] we draw the immediate conclusion that the level of basal ATPase activity is not necessarily relevant for quinoline drug resistance. Dd2 has higher V_{max} relative to 3D7, whereas 7G8 has lower. The degree of QN and MQ stimulation could be related to resistance to these specific compounds, since although the differences between Dd2 and 3D7 isoforms are quite small 3D7 PfMDR1 showed the greatest proportional drug stimulation of ATPase activity (see also [10]). Interestingly, strain 7G8 exhibits VPL insensitive CQR, whereas the strain Dd2 CQR phenotype is VPL reversible. Perhaps correspondingly, Dd2 PfMDR1 ATPase is inhibited by VPL, but the 7G8 isoform is not. Interestingly, the S1034C mutation is responsible for this loss of VPL sensitivity along with reduced catalytic efficiency (Table 1). However, most importantly, these results indicate no simple pattern among quinoline drug effects on ATPase activity for PfMDR1 isoforms found in these strains vs. the level or pattern of quinoline drug resistance exhibited by the strain. Also, PfMDR1 function is clearly quite different from that of its close homologue HuMDR1 (P-glycoprotein), since VPL has the strongest effects (up to 10-fold stimulation) of any compound on HuMDR1 ATPase [1], whereas effects are minor to nonexistent for PfMDR1 isoforms associated with resistance. We suggest these data indicate that the relative contribution of PfMDR1

to drug resistance is likely different for various CQR strains (e.g. Dd2 vs. 7G8).

The PfMDR1 isoforms that are currently found in CQR isolates may illustrate symbiotic relationships between mutant PfCRTs and mutant PfMDR1 that confer either preferred resistance patterns, fitness adaptations, or perhaps both. Perhaps the different V_{max} and K_m for Dd2 vs. 7G8 PfMDR1 isoforms, along with over expression levels (gene copy number), reflect fitness adaptations relevant to distinct Dd2 and 7G8 PfCRT mutations, whereas decreased quinoline drug stimulation of both Dd2 and 7G8 PfMDR1 ATPase activity reflects selection to further subtly modify quinoline drug resistance conferred by PfCRT.

These experiments with purified membranes allow more precise and accurate quantification of PfMDR1 ATPase activity and other molecular characteristics (e.g. perhaps binding of some drugs). However, obviously only transfection with PfMDR1 alleles into various parasite strains can provide precise quantification of their minor role in modulating drug resistance profiles. Put together, the two approaches are synergistic; transfection of some of the mutants constructed in the present work may offer one convenient way in which to test some concepts. Also, we note that although specific PfMDR1 codons are often sequenced for CQR isolates, and that some parallels between those data and the results in this paper can be drawn, more complete sequencing of PfMDR1 alleles as well as additional quantification of the frequency of 7G8 vs. Dd2 allele over expression in CQS vs. CQR isolates [9] will eventually be required to distinguish between current models generated by our in vitro work.

Acknowledgement

This work was supported by NIAID/NIH (RO1 AI056312).

References

[1] Kimura Y, Kioka N, Kato H, Matsuo M, Ueda K. Modulation of drug-stimulated ATPase activity of human MDR1/P-glycoprotein by cholesterol. Biochem J 2007;401:597–605.

[2] Sarkadi B, Price EM, Boucher RC, Germann UA, Scarborough GA. Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. J Biol Chem 1992;267:4854–8.

[3] Cowman AF, Karcz S, Galatis D, Culvenor JG. A P-glycoprotein homologue of *Plasmodium falciparum* is localized on the digestive vacuole. J Cell Biol 1991;113(5):1033–42.

[4] Wellems TE, Panton LJ, Gluzman IY, et al. Chloroquine resistance not linked to mdr-like genes in a *Plasmodium falciparum* cross. Nature 1990;345:253–5.

[5] Wellems TE, Walker-Jonah A, Panton LJ. Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7. Proc Natl Acad Sci 1991;88(8):3382–6.

[6] Fidock DA, Nomura T, Talley AK, et al. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell 2000;6(4):861–71.

[7] Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 2000;403(6772):906–9.

[8] Sidhu AB, Valderramos SG, Fidock DA. *pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. Mol Microbiol 2005;57(4):913–26.

[9] Price RN, Uhlemann AC, Brockman A, et al. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. Lancet 2004;364:438–47.

[10] Amoah LE, Lekostaj JK, Roepe PD. Heterologous expression and ATPase activity of wildtype vs. mutant PfMDR1 protein. Biochemistry 2007;46(20):6060–73.

[11] Welker E, Szabo K, Hollo Z, Muller M, Sarkadi B, Varadi A. Drug-stimulated ATPase activity of a deletion mutant of the human multidrug-resistance protein (MDR1). Biochem Biophys Res Commun 1995;216(2):602–9.

[12] Bruggemann EP, Germann UA, Gottesman MM, Pastan I. Two different regions of P-glycoprotein are photoaffinity labeled by azidopine. J Biol Chem 1989;264:15483–8.

[13] Bruggemann EP, Currier SJ, Gottesman MM, Pastan I. Characterization of the azidopine and vinblastine binding site of P-glycoprotein. J Biol Chem 1992;267:21020–6.

[14] Morris DI, Greenberger LM, Bruggemann EP, et al. Localization of the forskolin labeling sites to both halves of P-glycoprotein: similarity of the sites labeled by forskolin and prazosin. Mol Pharmacol 1994;46: 329–37.

[15] Frelet A, Klein M. Insight in eukaryotic ABC transporter function by mutation analysis. FEBS Lett 2006;580:1064–84.

[16] Dawson RJ, Locher KP. Structure of a bacterial multidrug ABC transporter. Nature 2006;443(7108):180–5.

[17] Wootton JC, Feng X, Ferdig MT, et al. Genetic diversity and chloroquine selective sweeps in *Plasmodium falciparum*. Nature 2002;418:320–3.

American Society of Tropical Medicine and Hygiene Annual Conference, November 4-8, 2007

Photoaffinity labeling of *P. falciparum* chloroquine resistance transporter (PfCRT)

Lekostaj JK, Natarajan JK, Amoah LE, Paguio MF, Wolf C & Roepe PD

Dept. of Chemistry, Dept. of Biochemistry, Cellular & Molecular Biology, and Center for Infectious Disease, Georgetown University

Several models for how amino acid substitutions in the *P. falciparum* chloroquine resistance transporter confer resistance to chloroquine (CQ) and other antimalarial drugs have been proposed. Further progress requires purified reconstituted PfCRT protein and more precise and convenient assays for analyzing possible interactions with CQ. We have designed and synthesized a per fluoro phenyl azido (pfpa) CQ analogue for PfCRT photolabelling studies. This probe (AzBCQ) places the pfpa group at the terminal aliphatic N of CQ, via a flexible 4 C ester linker, and also includes a convenient biotin tag. Using reconstituted proteoliposomes harboring partially purified PfCRT, we use AzBCQ and various amino acid mutations in PfCRT to probe the nature of the CQ binding site. Progress on competition vs. other drugs and on mapping the drug binding site(s) by mass spectrometry will be reported.